PHYSICAL ELIMINATION OF LYMPHOCYTES FROM HUMAN BONE MARROW

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'A new approach to prevention of graft versus host disease in allogeneic bone marrow transplantation?'

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Memento Mori

Aan Els, Moniek, Katrien en Lotje.

CONTENTS

CHAPTER I

INTRODUCTION

Induction therapy

Untreated adult patients with acute nonlymphoblastic leukemia (ANLL) have a median survival of 1.7 months after diagnosis (1). Intensive combination chemotherapeutic regimens including anthracycline antibiotics, Cytarabine and/or 6-Thioguanine have been reported to induce a complete remission in 60-85% of newly diagnosed adult ANLL (2,3,4,5). A recent report even claimed a 92% complete remission rate in children and young adults (age range: 2-30 years) (6). Patients who achieve a complete remission have a median survival of $\sim$ 100 weeks (7). Failure of achieving a complete remission is accompanied with a survival of less than 7 weeks (7).

Maintenance therapy

The median duration of complete remission following intensive chemoinduction therapy appeared to be 2-9 months without any maintenance therapy (8,9). Maintenance of remission by chemotherapy, immunotherapy or a combination of both failed to produce any substantial improvement of remission duration or survival (5,10,11). The median duration of remission in most recent investigations is 12-15 months (10,11,12), with the long-term survivorship among such patients being 10-25% (5,13). Recent introduction of more intensive maintenance therapy, implicating repetitive phases of severe bone marrow hypoplasia seems to have improved remission duration at least in young patients (6,9,14).

Allogeneic bone marrow transplantation

The failure of "standard" chemotherapy to induce long term disease free survival in ANLL may be due to inadequate reduction of the leukaemic cell number. Bone marrow transplantation (BMT) allows an intensification of the cytoreductive therapy, because the engrafted marrow rescues the patient from otherwise lethal bone marrow aplasia. Early attempts of BMT without matching for the major histocompatibility complex (MHC) were disappointing and failed with a few exceptions (15). MHC compatible siblings are commonly used as bone marrow donors, but unfortunately only 25-30% of the patients have a suitable donor. Initially BMT was performed in endstage acute leukemia with a 12% disease free two years survival (16). Initial mortality was due to advanced stage of illness, but recurrence of leukemia was a major cause of death 6-24 months after BMT. Relapses more than 2 year after BMT were a rare exception (16). This has led to trials of marrow transplantation in patients with ANLL in first remission. A disease free survival at two years of 50-80% has been reported (17,18,19,20). The chance of recurrent leukemia

in these patients has been extrapolated to be less than 10% (16). The major causes of early morbidity and mortality after BMT are due to direct toxicity of the bone marrow ablative therapy, increased susceptibility to infections and acute graft versus host disease (GVHD). Later mortality is mainly due to chronic GVHD and interstitial pneumonitis.

GRAFT VERSUS HOST DISEASE

Incidence of graft versus host disease

The incidence of graft versus host disease (GVHD) appears to increase with recipient age and it has been reported to occur in 60-80% of adult allogeneic graft recipients despite standard prophylaxis with Methotrexate (MTX) (21,22). The clinical picture of acute GVHD varies from a mild transient skin eruption to a fulminant syndrome of a generalised erythematous, maculopapular rash, enteropathy with severe diarrhoea and hepatitis often complicated by interstitial pneumonitis and fatal infections (23).
GVHD is conventionally explained by a cytotoxic effect of donor lymphocytes directed against host histocompatibility antigens (24,25) and especially directed towards antigens coded at non - HLA loci (26). Some authors suggested that the syndrome may result from an imbalance of T-lymphocyte subsets, resulting in a proliferation of cytotoxic T-lymphocytes, normally controlled by suppressor T-cells, but conflicting reports have been published (27,28,29,30).

Prevention of graft versus host disease

The usual approach of preventing GVHD is suppression of the immunocompetent cells after bone marrow infusion. Administration of Methotrexate for 100 days after BMT reduced the incidence and severity of GVHD and it is most commonly used according to the Seattle protocol (21). However, still 50-70% of patients receiving allogeneic bone marrow from identical siblings develop GVHD (21). Recently, antilymphocyte globulin (31) and Cyclosporin A (32,33) have shown to prevent and/or to delay severe forms of GVHD. The value of these approaches has not been confirmed in randomised studies, but unpublished studies (Thomas) have not shown superior results when Cyclosporin A was compared to Methotrexate.
Several studies in animals have shown a clear association between the number of T-lymphocytes present in the grafted material and severity of GVHD (25). Removal of immunologically competent mature T-lymphocytes from the marrow graft has been exploited in an attempt to reduce the incidence and/or severity of GVHD. Using differences in cell density, albumin gradient centrifugation has been performed, but the limited yield of hematopoietic stem cells has restricted clinical application to children (34). In vitro

incubation of donor marrow with specific, purified antisera raised in rabbits to human thymocytes has been reported by Rodt et al (35). Unfortunately it is difficult to prepare and standardize this reagent in sufficient quantity. In vitro opsonisation of donor T-lymphocytes with monoclonal antibodies with "pan-T" specificity has been attempted, but conflicting results appeared in 2 reports (36,37). Depletion of donor T- lymphocytes from HLA-A and -B nonidentical parenteral marrow before grafting by differential agglutination with the lectin soybean agglutin followed by rosetting procedures resulted in a successful bone marrow repopulation of an infant with acute lymphoblastic leukaemia(38).

Physical separation of human bone marrow

Initially, buoyant density gradient centrifugation systems were used for physical fractionation of complex cell mixtures like human bone marrow. Discontinuous albumin gradients were employed by Dicke et al.to eliminate lymphocytes from the hematopoietic stem cells (39). Disadvantages of albumin density gradients are problems in controlling osmolarity of different batches of bovine serum albumin (BSA) and the aphysiological pH of 5.1 of the gradients (40, 41). Isopaque-Ficoll^{R} density gradients (40,42) have the disadvantage of high viscosity (43) and selective retention of B-lymphocytes (44). Polyvinylpyrrolidone coated silica gel (Percoll^{R}) has been introduced more recently (45). Density gradients of physiological osmolarity are easily obtained due to the low osmolarity of Percoll. Additional advantages are: high stability of the gradient, constant physico-chemical properties between different batches and low viscosity (41).

Several methods have been used to separate human bone marrow cells according to cell size. Velocity gradient sedimentation at unit gravity in different types of sedimentation chambers has been described, including one in our laboratory (46,47). The main disadvantages are the limited number of cells (2x 10^{8}) that can be separated and the long duration of the separation procedure (3-5 hours) (40).
Counterflow centrifugation (CC) exposes cells to a centrifugal force and an opposing flow force (48,49,50). Cell separation takes place in a small chamber, (4.2 ml) and cells leave the chamber, mainly depending on cell size, by decreasing the rotor speed or by increasing the flow rate. After several adaptations (51,52) a reliable separation system was obtained with important advantages: short duration of the separation procedure, high capacity, good recovery, excellent cell viability and homogeneity of cell size in the different fractions, related to cell cycle phase within the various subpopulations (53).

PURPOSE OF THE STUDY AND OUTLINE OF INVESTIGATIONS

The initial purpose of this study was the development of separation systems in order to obtain bone marrow fractions enriched for hematopoietic stemcells. These fractionations were performed with isopycnic gradient centrifugation and counterflow centrifugation. When it appeared feasable to separate lymphocytes almost completely from the hematopoietic progenitor cells, efforts were directed to a new approach to prevention of graft versus host disease (GVHD) by elimination of the lymphocytes from the bone marrow grafts, using a combination of density centrifugation and counterflow elutriation.

For assessment of the viability and quantification of numbers of stem cells in the different fractions in vitro clonogenic assays were developed for the committed progenitor cells and pluripotent stem cells (chapter II).
Isopycnic gradient centrifugation in Percoll was developed to obtain a low density fraction devoid of erythrocytes and most mature granulocytes without any substantial loss of committed stem cells (chapter III).
Bone marrow cells of varying density were separated in different fractions by counterflow elutriation and analysed for morphology, in vitro clonogenic cells and T-lymphocyte subpopulations (chapter IV).
Counterflow centrifugation separates cell populations mainly depending on cell size and within each cell population according to the cell cycle phase (chapter V).
Low density cells from normal human bone marrow were fractionated in two fractions by means of counterflow centrifugation. These two fractions were analysed in vitro for the presence of the pluripotent stem cells and for the mitogenic reactivity (chapter VI).
Bone marrow from 6 donors for allogeneic bone marrow transplantation was separated by density gradient centrifugation followed by counterflow elutriation. In vitro culture results, immunological evaluation, in vivo bone marrow repopulation capacity in 6 patients with acute leukemia in first remission and the incidence of GVHD are described in chapter VII.

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CHAPTER II

QUANTIFICATION OF PLURIPOTENT AND COMMITTED HEMATOPOIETIC CLONOGENIC CELLS IN HUMAN BONE MARROW

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SUMMARY

Accurate and reproducable in vitro assays of clonogenic stem cells are mandatory in order to evaluate the accurate number of hematopoietic stem cells (HSC) after manipulation of human bone marrow. Media obtained from placental tissue conditioned in the presence of endotoxin provided a CSF-source with high colony stimulating activity and almost no inhibitory activity. The use of frozen marrow samples from one healthy donor allowed a standardization of media and stimulating factors. Estimation of blood contamination in bone marrow samples by a simple method based upon the assumption that almost all hemoglobin in a bone marrow sample originates from blood showed a significant negative correlation between blood contamination and the number of BFU-E and CFU-GM present in bone marrow. The normal values of CFU-GM, BFU-E and CFU-GEMM after exclusion of bone marrow samples contaminated with more than 15% peripheral nucleated cells are presented.

INTRODUCTION

Haematopoiesis implies a continuous production of erythrocytes, granulocytes, thrombocytes, lymphoid cells, osteoclasts and various stromal cells. The general concept is that cell production takes place at three levels. The first level is the stem cell population, which is pluripotential in the sense that it gives rise to different cell populations and it is capable of self-renewal. The second level is a first differentiation step producing progenitor cell populations. These are morphologically unrecognizable, but they can be demonstrated by in vitro colony formation under appropriate culture conditions. The third level is achieved during a second differentiation step at which the progenitor cell gives rise to a morphologically recognizable blast cell population (precursor-population). This population differentiates further by several cell divisions till an end stage cell occurs, which has lost its proliferation potential and matures till functionality. It is clear that mature cells have a definite life span and have to be replaced continuously from the stem cell pool.

Experimental data in mice (1) suggest that about 10% of the stem cells are differentiating per day. One hemopoietic stem cell (HSC) entering the amplification compartment produces 60 progenitor cells and about 1000 granulocytes ($t_{1/2} < 1$ day) or 200.000 erythrocytes ($t_{1/2} \sim 100$ days) in the peripheral blood. This means that the ratio of murine stem cells to progenitor cells to recognizable nucleated cells in bone marrow will be: 10:60:4000. In other words about 0.25% of the bone marrow cells are real stem cells and 1.5% progenitor cells, assuming that bone marrow is not contaminated with blood.

The development of semisolid culture systems has allowed probably all hemopoietic progenitor and stem cells to be cloned in vitro with relative high plating efficiencies. Cell proliferation in vitro depends on presence of specific growth regulators, such as colony stimulating factor (CSF) for the granulocyte-macrophage proliferation and erythropoietin (EPO) for the erythroid proliferation (2). Pluripotent hemopoietic stem cells (3) proliferate and differentiate in the presence of medium conditioned by phytohemagglutinin stimulated leukocytes: PHA-LCM (4). Fauser and Messner (5) observed the presence of eosinophilic and neutrophilic granulocytes, erythroblasts, macrophages and megakaryocytes in mixed colonies, originating from pluripotent stem cells: colony forming unit - granulocyte - erythroblast - macrophage - megakaryocyte (CFU-GEMM). Recently, also the presence of T-lymphocytes in these mixed colonies was demonstrated (6). Limited self renewal capacity of CFU-GEMM has been reported (7), making the CFU-GEMM a strong candidate for the HSC. The low number of these cells in human bone marrow ($\sim$0.01%) hampers a reliable quantification of stem cells present in a bone marrow fraction.

The committed granulocyte - macrophage/monocyte progenitor cell (CFU-GM) proliferates in vitro to form colonies of granulocytes (eosinophilic and neutrophilic) and macrophages (8,9). These cells,having a relatively high proliferation rate, could be separated partially from the murine HSC by velocity sedimentation and isopycnic centrifugation techniques (10). None the less the number of CFU-GM present in cell fractions has proven to provide a reliable indication of the capacity to regenerate hematopoiesis (11). The erythroid progenitor population consists of two distinct cell types: a more immature stem cell, called the erythroid burst forming unit (BFU-E) and a more differentiated descendant from the BFU-E: erythroid colony forming unit (CFU-E) (12). Proliferation of BFU-E requires stimulation by a burst promoting activity (BPA): a factor present in leukocyte conditioned medium (13) and EPO; growth of CFU-E depends only on the presence of EPO.

MATERIALS AND METHODS

Preparation of bone marrow cells

Normal bone marrow, obtained from patients undergoing cardiac surgery or from healthy bone marrow donors, is collected in buffered acid-citrate dextrose (pH 7.0). Bone marrow particles are disrupted by repeated aspirations through 16 gauge needles and the suspension is filtered through a nylon filter (pore- size 70 µm). The cells are washed in Ca^{++}/Mg^{++} -free Hanks balanced salt solution (HBSS) for removal of plasma and fat and resuspended in HBSS containing 5% (v/v) fetal calf serum (FCS). A number of

150-200 x 10^6 nucleated cells in 35 ml suspension is layered on 15 ml Ficoll-Isopaque (specific density : 1.085 g/ml; pH 6.6; osmolality : 300 mosm/kg) and centrifuged at 500 g and 18° C for 20 minutes to remove the red cells (14). The nucleated cells at the interphase are collected, washed and resuspended in 3-5 ml FCS. This fraction contains 76 ± 4% of the original nucleated cells, 49 ± 12% of the mature myeloid cells and 105 ± 21% of the CFU-GM (15).

Preparation of conditioned media

Human placenta conditioned medium (HPCM) is prepared according to the method described by Schlunk and Schleyer (16). In some experiments the placentas are conditioned in the presence of endotoxin: lipopolysaccharide W (LPS) from E-Coli (3120-25, Difco): 10µg/ml (LPS-HPCM) (17).
Human leukocyte conditioned medium (HLCM) is prepared from human peripheral blood leukocytes immobilized in 0.5% agar for 7 days as described by Iscove et al. (18). Venous blood is obtained from normal volunteers or patients with hemochromatosis and collected in preservative free heparin. Human mononuclear peripheral blood leukocytes are prepared by isopycnic density cut separation through Ficoll-Isopaque (d: 1.077 g/ml). Human mononuclear conditioned medium is prepared in the presence of phytohemagglutinin(Wellcome MR10): 90 µg/ml for 7 days as described by Aye (4) (PHA-HMCM).

Granulocyte/macrophage colony forming cells (CFU-GM)-assay (19)

All cultures are performed in 2 ml volumes in 35 mm^2 plastic Petri dishes (Costar). Dulbecco's Modified Eagle's Medium is used with 20 % heat inactivated FCS from a preselected batch in 0.3 % Bacto-agar(Difco). Nucleated cells ($2x10^5$) are seeded per plate with 4% (w/v) colony stimulating factor (CSF) obtained from placentas (LPS-HPCM) or CSF derived from a GCT-cell line (Gibco) (20).Duplicate cultures are incubated at 37° C in a fully humidified atmosphere of 5 % CO_2 in air for 10-12 days. Cultures are scored at 20-40 x magnification. Aggregates consisting of 40 and more cells are scored as colonies.

Erythroid colony forming cells (BFU-E/CFU-E)-assay.

The basic culture technique is adapted from the method of Iscove et al.(21). The cell suspensions at a concentration of 1 x 10^5/ml are plated in a substantially modified Dulbecco's Medium, prepared from powder (IMDM, Gibco formula 78 - 5220) (22), to which is added 0.8% methylcellulose (Fluka, Schweiz), 10^{-4}M thioglycerol, 10 % deionized bovine serum albumin (12) 20 % heat inactivated foetal calf serum of a preselected batch (Rehatuin), 20 %

leukocyte conditioned medium (14) and 2 Units sheep erythropoietin/ml (Step III, Connaught Lab, Willowdale Ont. Can.). One-tenth milliliters of this suspension are placed in flat bottomed microwells (Limbro, Titertex). Orange-to-red colonies of at least eight cells are scored as CFU-E in four microwells on the 7th day of culture. Orange-to-red bursts of at least three subclusters or one single colony of more than 300 cells are scored as BFU-E in the other four wells on day fourteen of the culture.

Pluripotent colony forming cells (CFU-GEMM)-assay

The basic culture conditions were outlined by Fauser and Messner (5), but some modifications according to Ash et al. (7) are followed. Cell suspensions at a concentration of 1 x 10^5/ml are plated in Iscove's modified Dulbecco's medium containing methylcellulose (Fluka, Schweiz) as viscous support (0.9% final concentration), 20% heat inactivated FCS from a preselected batch (Rehatuin), 5% PHA-HMCM, 10% deionized bovine serum albumin, 10^{-4}M thioglycerol and 1.5 Unit sheep erythropoetin (Step III EPO; Connaught Lab. Willowdale Ont. Can). Duplicate 0.5 ml aliquots are cultured in 16 mm^2wells (Cluster^{24}, Costar) and mixed colonies are identified on day 16 of culture by their composite appearance. Individual colonies are removed by micropipetting into thin-tipped Pasteur pipettes, transferred onto slides and forcefully air-blown to spread the cells. Slides are stained with May Grünwald-Giemsa to assess their morphological composition.

RESULTS

Colony stimulating and inhibiting factors in HPCM

The colony stimulating activity (CSA) of six different batches of HPCM was compared to that of a commercially available batch from a giant cell tumor cell line (GCT-CSF). The results are listed in table I and show the variable and often suboptimal CSA. Conditioning in the presence of E. Coli endotoxin (LPS) yielded increased levels of CSA.
Different batches of HPCM were tested for the presence of stimulating and inhibiting factors by performing CFU-GM-assays with varying dilutions of conditioned medium. Increasing quantities of HPCM conditioned without LPS resulted in considerable inhibition of colony formation (table II). This inhibition was absent or less apparent if HPCM was prepared in the presence of endotoxin. For these reasons it was decided to use in this study only HPCM conditioned in the presence of endotoxin at a concentration of 4% v/v.

Batch HPCM	LPS	CSA%
1	-	78 ± 24*
2	-	42 ± 8
3	-	72 ± 32
4	-	58 ± 12
5	-	111 ± 51
5	+	140 ± 54
6	-	118 ± 10
6	+	144 ± 18

* mean ± SD (n=4)

Table I.

Colony stimulating activity (CSA) of different batches human placenta conditioned medium (HPCM) compared to CSA of one batch GCT - CSF. CSA of GCT - CSF is put at 100 %. Two batches were also prepared in presence of LPS.

Batch HPCM		Volume % of HPCM in CFU-GM assay					
		40	30	20	10	4	2
1	-LPS	19	69	113	100	77	-
2	-LPS	1	10	70	100	77	24
5	-LPS	86	86	107	100	98	-
6	-LPS	0	15	93	100	142	151
5	+LPS	103	97	91	100	103	96
6	+LPS	75	99	111	100	117	114

Table II.

Colony stimulating and inhibiting factors present in HPCM. Influence of presence of endotoxin (LPS). Each batch of HPCM was tested 3-6 times. Results are expressed as mean percentage of the number of CFU-GM obtained with 10 % w/v HPCM. Batch nrs correspond with those of table I.

Frozen marrow serving as standard control

The use of different batches FCS, conditioned media and the varying culture conditions in the CO_2 incubators asks for an internal standard control. Therefore a batch of 2 ml ampoules (Nunc, Intermed) with normal human bone marrow at a concentration of 1 x 10^6 nucleated cells per ml autologous plasma with 10 % DMSO was stored in liquid nitrogen as described before (24). One single batch of ampoules containing normal bone marrow from one bone marrow donor was used during the observation period. CFU-GM were cultured on 67 occasions: normal growth was observed 56 times: 18 ± 5 CFU-GM/10^5 nucleated cells; subnormal growth: 4 times and no growth 7 times due to an inadequate batch of culture medium. BFU-E were cultured on 24 occasions: normal growth was observed 16 times: 39 ± 11 BFU-E/10^5 nucleated cells; subnormal growth: 3 times and 5 cultures were found to have dried out. The variation from month to month during the observation period was acceptable (figure 1).

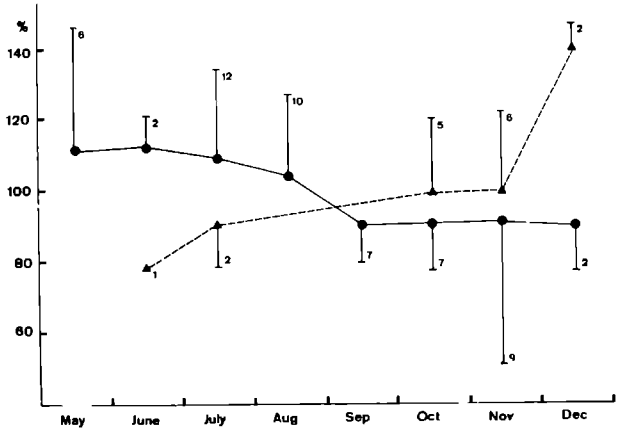

Figure 1
The average number of CFU-GM (●——●) and BFU-E (▲---▲) per month of one single batch of ampoules containing frozen normal bone marrow cells. The average number of CFU-GM and BFU-E of cultures with normal growth was put 100%. Numbers refer to number of cultures of that particular month with normal growth and each vertical bar represents one standard deviation of the mean of that month.

Normal values of pluripotent stem cells and progenitor cells
Influence of peripheral blood contamination

The number of circulating CFU-GM and BFU-E per 2 x 10^5 nucleated cells in human blood is less than one percent of the number of progenitor cells present in bone marrow. Bone marrow contaminated with a high number of peripheral nucleated cells will exhibit a low number of colony forming cells. In order to determine the normal values of committed and pluripotent stem cells, the blood contamination was compared with the actual number of committed erythroid and myeloid progenitor cells in bone marrow samples. The contamination of peripheral nucleated cells in bone marrow aspirates was calculated as described by Holdrinet et al. (23) with use of the formula:

$$\text{Fraction Bl} = \frac{Hb_{BM} \times NC_{Bl}}{Hb_{Bl} \times NC_{BM}}$$

where Hb=hemoglobin, BM=bone marrow, Bl=blood and NC=nucleated cells. CFU-GM from 65 normal bone marrow samples are plotted against the peripheral blood contamination in figure 2. The calculated correlation factor r was: - 0.51 ($p < 0.001$).
The relation of CFU-E and BFU-E with blood contamination is presented in figure 3 . Normal values of erythroid, myeloid and pluripotent clonogenic cells are shown in tableIII.Bone marrow samples with more than 15% of the nucleated cells originating from blood admixture were excluded.

CFU-GM	55 ± 19	n=48
BFU-E	124 ± 45	n=22
CFU-E	344 ± 131	n=13
CFU-GEMM	11 ± 6	n= 8

Table III.

Normal values of bone marrow samples obtained from hematologically normal persons. Bone marrow samples with more than 15 percent blood contamination were excluded. The results are expressed as mean number ± one SD per 10^5 nucleated cells. n is number of observations.

The number of CFU-GM in normal bone marrow samples observed in 3 month periods during 3 years are presented in figure 4.
GCT-CSF was used until March 1981 and two batches of HPCM+LPS since then.

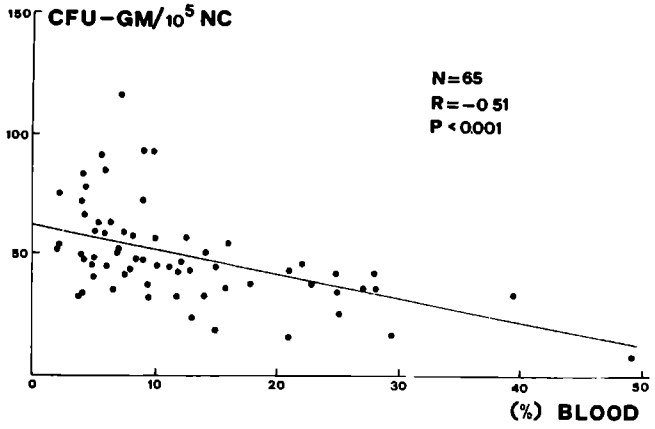

Figure 2
The influence of peripheral nucleated cell (NC) contamination on the number of CFU-GM in 65 bone marrow aspirates of hematologically normal persons. The correlation factor (r) was: - 0.51 corresponding with a $p < 0.001$.

DISCUSSION

Manipulation of human bone marrow by physical separation procedures requires an accurate monitoring of the survival of the HSC in order to predict bone marrow repopulation capacity. Until recently investigations of this nature were limited to committed progenitor cell populations (CFU-GM and BFU-E), which form the immediate progeny of the pluripotent hematopoietic stem cells. The number of myeloid progenitor cells has been shown to be of value in predicting hematopoietic recovery after bone marrow ablative therapy (11). However, several problems concerning standardization remained to be solved.

Firstly, the fluctuating activity of CSF depending on the source and activity of conditioned media. The original CSF - source as described by Pike and Robinson (18) was formed by peripheral blood leukocytes immobilized in a feeder layer of 0.5 % agar. The colony stimulating activity (CSA) varied considerably, even when blood from one single donor was used (24).

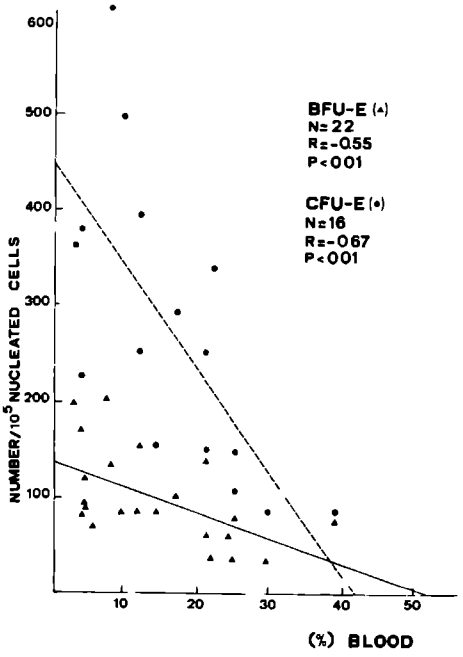

Figure 3
The influence of peripheral nucleated cell (NC) contamination on the number of erythroid progenitor cells in bone marrow aspirates of hematologically normal persons.
The correlation factor for the BFU-E was - 0.55 and for the CFU-E - 0.67 corresponding with a $p < 0.01$.

Conditioned media from placenta (26) provide large quantities of CSF, but although culture conditions were improved (15) the level of CSA in different batches appeared to be suboptimal (table I). Moreover dose-response curves showed the presence of inhibitors in most batches (table II). Low CSA-levels and presence of inhibitors may result in fluctuating datas due to direct or indirect interaction through monocytes, T-lymphocytes, and mature granulocytes, which are present in high number in several bone marrow fractions. Medium conditioned in the presence of endotoxin (HPCM+LPS) showed a higher CSA-level and a low level of inhibitors. An additional

advantage with this CSF-source was the complete recovery of CFU-GM, which could be achieved after cryopreservation in liquid nitrogen, as we described before (25). This contrasted the suboptimal recovery obtained with other CSF-sources like HPCM-CSF, GCT-CSF and PHA-HMCM (25).

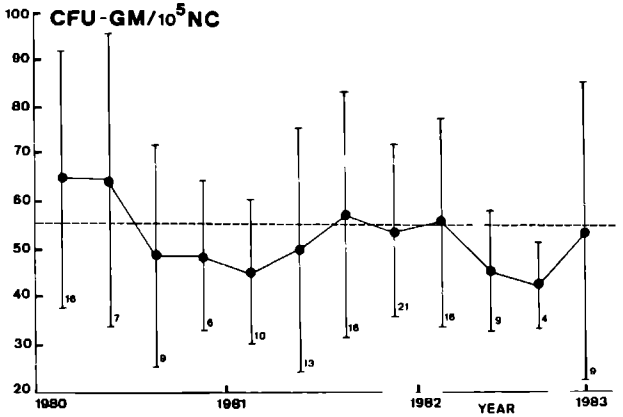

Figure 4
The mean number of CFU-GM (± SD) per trimester in bone marrow aspirates of hematologically normal persons. Numbers refer to number of cultures of that particular month. Bone marrow aspirates with more than 15% blood contamination were excluded.

The second problem of standardization is due to the wide variations in the number of CFU-GM in normal human bone marrow, even within the same donor. The use of frozen marrow samples for standardization of media, CSF and culture conditions can overcome this problem. We were able to show that one batch of normal bone marrow samples resulted in reproducible numbers of CFU-GM and BFU-E (fig. 1). By consistent application of this internal standard, the reproducability of the in vitro cultures was improved.
Contamination of marrow aspirates with blood may also cause errors when the number of colonies are related only to 10^5 nucleated cells. Complicated methods to determine blood contamination of bone marrow have been advocated (27), but Holdrinet et al. developed a practical formula, based upon experiments with labelled red cells, that showed that nearly all hemoglobin present in a bone marrow aspirate originates from blood contamination (23). Taking into account the obvious interindividual variation in normal bone marrow, this method showed a clear negative correlation between the number

of clonogenic cells per 10^5 nucleated cells and the amount of blood contamination (figs. 2 and 3). The normal values of clonogenic cells were calculated after exclusion of bone marrow samples contaminated with more than 15 % peripheral nucleated cells.

In conclusion: standardization of in vitro culture assays is crucial for the evaluation of bone marrow fractions obtained by different separation techniques. This could be accomplished by using a highly active source of CSF, frozen bone marrow samples as internal control and estimation of peripheral nucleated cell contamination.

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CHAPTER III

ENRICHTMENT OF MYELOID CLONOGENIC CELLS BY ISOPYCNIC DENSITY EQUILIBRIUM CENTRIFUGATION IN PERCOLL GRADIENTS AND COUNTERFLOW CENTRIFUGATION

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SUMMARY

Continuous density gradients made from polyvinyl-pyrrolidone-coated silica gel (Percoll) were used to obtain fractions enriched with human myeloid progenitor cells. Isopycnic separation with sample application to the bottom of the gradient (flotation) improved the recovery and yielded a fraction 25 times enriched for the CFU-GM. The density of this fraction was 1.0615 g/ml. Lymphocytes accounted for almost 10% of this fraction. Removal of the small sized lymphocytes was accomplished by counterflow centrifugation with continuous cell-size monitoring of the effluent by light scatter. The obtained large cell fraction containing all myeloid progenitor cells was subsequently subjected to isopycnic flotation centrifugation. Only 0.2% of the original number of lymphocytes contaminated the two fractions with the highest number of clonogenic cells. Physical separation methods based on counterflow centrifugation and isopycnic centrifugation may offer a new approach to the elimination of lymphocytes from human bone marrow in order to prevent or mitigate GVHD in allogeneic bone marrow transplantation.

INTRODUCTION

Normal human bone marrow contains committed myeloid progenitor cells (CFU-GM). These cells represent the immediate progeny of the pluripotent hematopoietic stem cells (1). The number of CFU-GM in cell fractions has proven to provide a reliable indication of the capacity to regenerate hematopoiesis (2). The purpose of this study is the development of a method to obtain a fraction of human bone marrow, highly enriched for myeloid progenitor cells and almost devoid of lymphoid cells. The density (3) and the size (4) of cells are the main physical characteristics, which can be used for separation of heterogeneous cell populations. Density gradient centrifugation is mainly used to fractionate bone marrow according to the various stages of maturation (5). Colloidal silica coated with polyvinyl-pyrrolidone (Percoll) has been claimed to provide gradients superior to bovine serum albumin and Ficoll due to high stability, constant physico-chemical properties, low viscosity and easy preparation of isotonic solutions with each desired pH (6).
Counterflow centrifugation (elutriation) separates cells mainly according to their size (7, 8). This method provides the possibility of separating small sized lymphocytes from the larger sized myeloid progenitor cells (9).
In this study the recoveries of nucleated cells and myeloid progenitor cells have been studied as obtained after isopycnic separation with sample application to the top or the bottom of the gradient (flotation). The density and cell composition in the fractions with the highest enrichment of progenitor cells, obtained before and after removal of the small sized lymphocytes by means of counterflow centrifugation, is described in detail.

MATERIALS AND METHODS

Preparation of bone marrow cells

Normal bone marrow from patients undergoing cardiac surgery is collected in buffered acid-citrate dextrose (pH 7.0). Bone marrow particles are disrupted by repeated aspirations through 16-gauge needles and the suspension is filtered through a nylon filter (pore size 70 μm). The cells are washed in Ca++/Mg++- free Hanks' balanced salt solution (HBSS) for removal of plasma and fat and resuspended in HBSS containing 5% (w/v) fetal calf serum (FCS).

Preparation of isotonic percoll density gradient

An isotonic stock solution of Percoll (Pharmacia, Uppsala, Sweden) is made by mixing 9 parts of Percoll with 1 part of 10 x concentrated HBSS (osmolality 285 mosm/kg). Solutions with the desired density (d) are made by mixing X ml of the Percoll stock solution (density a) with Y ml of isotonic HBSS (density b) according to the formula:

$$\frac{X}{Y} = \frac{a - d}{d - b}$$

Density is measured by reading the refractive index (RI) and converting the RI values to density from a standard curve made for Percoll suspended in HBSS. The pH is adjusted to 7.4 by flushing the suspensions with 7% CO_2 in air.

Preparation of continuous linear density gradients

Linear gradients of 35 ml in 50 ml centrifuge tubes are prepared as described by Loos and Roos (3) with the use of a conical bore universal gradient mixer (Buckler Instruments, Fort Lee, N.J., USA). The gradients are introduced under a 5 ml solution with the same density as the top end of the gradient to avoid perturbation of the linearity. The linearity is always checked by reading the RIs of the collected fractions. The gradients, with caps tightly screwed, are stored at 4^0C until used. The linearity is maintained for at least 3 months, and is not disturbed by centrifugation.

Isopycnic sedimentation centrifugation

A number of (100-150) x 10^6 nucleated bone marrow cells in 10 ml of suspension are carefully layered on the linear gradient and centrifuged at 500 g and 18^0 for 30 min. After sedimentation, a stainless-steel needle fixed

in a gradient sampler is introduced from above and fractions are collected by a peristaltic pump at a rate of 2 ml/min.

Isopycnic flotation centrifugation

A 10 ml suspension is prepared with a density equal to the bottom end of the gradient by mixing 6.7 ml Percoll stock solution, 2.3 ml HBSS, 1 ml FCS and 100-150 x 10^6 bone marrow cells. The total cell volume of this suspension influences the density of the suspension. The desired density is obtained by the addition of a small volume of HBSS to correct the refractive index. The cell suspension is layered under the gradient using a syringe fitted with a blunted, large-bore needle. Centrifugation and collection of the fractions are identical to the sedimentation centrifugation.

Counterflow centrifugation

Counterflow centrifugation (CC) is performed with a Beckman J2- 21C refrigerated centrifuge equipped with a Beckman JE-6 elutriation rotor with a standard separation chamber (Beckman Instruments Inc., Palo Alto, Calif.). A constant flow rate is obtained by a peristaltic pump (Masterflex-Cole-Parmer Instruments, Chicago, Ill., USA). The pump is connected to a pulse flattening air chamber. The elutriation system is disinfected by overnight incubation with 70% ethanol. HBSS supplemented with 1% (w/v) heat-inactivated FCS is used as elutriation medium, and maintained at 18°C. The output of the elutriator is continuously sampled by means of a T-drain and analyzed for cell number and light scatter by the electro-optical unit of a Hematolog D (Technicon Instrument Corp., Tarrytown, N.J., USA). The light scatter signals are accumulated and displayed on a ND-600 multichannel analyzer (Nuclear Data Inc., Schaumberg, Ill., USA) (9). The specimens are introduced by means of an infusor pump (0.3 ml/min) into the elutriator rotor spinning at 2.200 rpm and a counter flow rate of 9 ml/min. Only thrombocytes, erythrocytes and small lymphocytes are eluted at this flow rate. The rotor speed is maintained at 2.200 rpm throughout the procedure and the counterflow rate is increased stepwise 1-2 ml/min whenever the number of elutriated cells detected by the electro-optical system falls below 100 cells/s.

Granulocyte/macrophage colony forming cells (CFU-GM)-assay

All cultures are performed in 2-ml vol in 35 -mm^2 plastic Petri dishes (Costar). Dulbecco's modified Eagle's medium (Flow) is used with 20% heat-inactivated FCS from a preselected batch in 0.3% Bacto-agar (Difco). Nucleated cells (2 x 10^5 or less) are seeded per plate with 4% (w/v) colony stimulating factor derived from human placenta conditioned medium (HPCM)

(10,11). Duplicate cultures are incubated at 37^0C in a fully humidified atmosphere of 5% CO_2 in air for 10-12 days. Cultures are scored at 20-40 x magnification. Aggregates consisting of 40 and more cells are scored as colonies, and aggregates of 3-40 cells as clusters.

Morphological studies

Cell counts are performed with a Coulter Counter (Model ZF; Coulter Electronics, Hialeah, Fla., USA). Slides of the fractions obtained by density sedimentation and elutriation are made with a cytocentrifuge and stained with May Grünwald- Giemsa. Differential counting of 400 cells is carried out by a single investigator (G.B.).

Statistics

The Wilcoxon sign rank test is used to calculate statistical differences in paired and unpaired observations. All results are expressed as mean ± one SD.

RESULTS

Isopycnic centrifugation in linear gradients with a density ranging from 1.050 to 1.085 g/ml

Linear gradients with a density ranging from 1.050 to 1.085 g/ml were prepared, and after centrifugation fractions of 3 ml were collected as described under 'Methods'. The recoveries of the nucleated cells and the myeloid progenitor cells were only 70% (table I) probably due to entrapment of these cells by the erythrocytes in the pellet. Therefore, flotation

	Flotation (n = 13)		Sedimentation (n = 7)	
	gradient	pellet	gradient	pellet
Nucleated cell	80.2 ± 10 6	6.0 ± 2.9	71.9 ± 13.0	11.3 ± 5.1
CFU-GM	81.9 ± 12.1	1.0 ± 1.2	69.0 ± 9.8	ND
Cluster	97 6 ± 19.9	1.0 ± 1.5	86.2 ± 8.0	ND

ND = Not determined.

Table I

Comparison of the recovery (%) after two wash steps in the fractions of linear gradients after isopycnic sedimentation and flotation centrifugation (d = 1 050-1.059 g/ml)

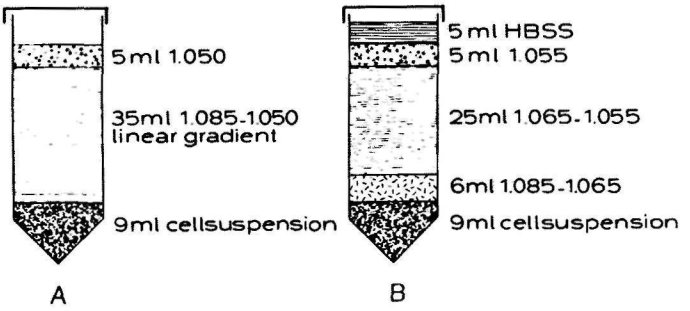

Fig. 1. Continuous density gradients with sample application under the linear gradient in a cell suspension with a density of 1.086-1.085 g/ml. 1a: linear gradient from 1.085 to 1.050 g/ml; 1b: shallow linear gradient from 1.065 to 1.055 g/ml (for details see: "Materials and Methods").

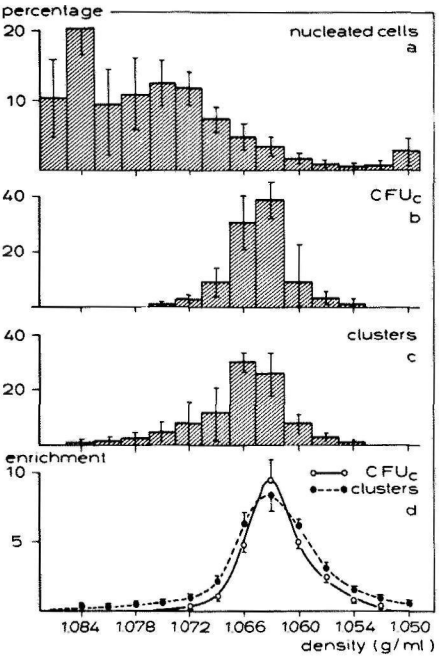

Fig. 2. Isopycnic centrifugation of normal bone marrow from 6 patients in continuous linear gradients with a density ranging from 1.050 to 1.086 g/ml. a-c Recovery of the nucleated (a) and the clonogenic cells (b,c) per fraction expressed as percentage of number in the loaded sample. d Enrichment of CFU-GM (O——O) and cluster-forming cells (O-----O) per fraction. The loaded sample was put at 100%.

centrifugation was tried with cell suspensions made in Percoll with a density equal to the highest density in the linear gradient, introduced at the bottom (fig. 1a). These flotation experiments resulted in a considerably better yield as compared to sedimentation centrifugation, although it did not reach significancy due to the low number of experiments. The loss of clonogenic cells in the pellet fractions was only minimal. Distribution and enrichment of the clonogenic cells in the collected fractions from six different bone marrow samples is presented in figure 2. A sharp peak of clonogenic cells was obtained: the absolute and relative peak of CFU-GM was found in the fraction with a density of 1.0615 g/ml. The peak fractions contained only 3.5% of the nucleated cells, 41% of the CFU-GM and 35% of the cluster-forming cells. The relative peak of the cluster-forming cells was found in the same fraction, but the absolute peak was shifted to the fraction with a density of 1.0645 g/ml. The total number of CFU-GM in the fraction with a density of 1.0615 g/ml was statistically higher than the number of clusters ($p<0.05$), although the density of the whole CFU-GM population was not statistically different from that of the cluster-forming cells. The recovery of these cells in the peak fraction and the two adjacent fractions is shown in table II. Myeloblasts form the major cell population in peak fractions. The morphological composition of the clonogenic cell peak fraction is presented in table IIIA.

	Peak fraction d = 1 064-1 061 g/ml	Peak fraction ± one fraction d = 1 067-1 058 g/ml
Nucleated cell	3.5 ± 1 5	10 0 ± 3 6
CFU-GM	41.1 ± 12 0	81 7 ± 13.7
Cluster	35.0 ± 8.4	70.6 ± 11.1

Table II.

Recovery (%) of nucleated cells and progenitor cells in peak fractions obtained from continuous linear gradients (density ranging from 1.085 to 1.050 g/ml) (n = 6).

Isopycnic centrifugation in linear gradients with a density ranging from 1.055 to 1.065 g/ml

The majority of the clonogenic cells was found in the fractions with densities ranging from 1.055 to 1.065 g/ml (fig. 2b,c). A shallow density gradient was developed to determine more exactly the density of the CFU-GM and the morphological composition of these fractions. Figure 1b gives some details of the gradient. A linear gradient of 25 ml with a density from 1.065 to 1.055 was introduced below 5 ml of a Percoll solution with a density of 1.055 g/ml. A 6-ml linear gradient with a density from 1.065 to 1.085 g/ml and

	Peak fraction[1]	BM (A)	Peak fraction[2]	BM (B)
Myeloblast	27.1 ± 6.0	1.7 ± 1.0	29.3 ± 8.4	2.5 ± 0.6
Promyelocyte	10.9 ± 2.4	0.7 ± 1.0	8.3 ± 5.2	1.5 ± 1.3
Myelocyte	12.0 ± 6.0	5.1 ± 3.2	9.8 ± 1.5	6.5 ± 2.5
Metamyelocyte	6.1 ± 2.9	10.3 ± 3.3	3.0 ± 2.2	8.0 ± 1.2
Mature granulocyte	4.7 ± 1.5	51.7 ± 7.3	3.8 ± 2.2	44.3 ± 12.6
Lymphocyte	13.0 ± 5.4	8.0 ± 2.8	8.8 ± 4.0	13.3 ± 6.8
Monocyte	10.0 ± 4.5	3.0 ± 1.3	15.8 ± 8.5	2.5 ± 1.0
Plasma cell	4.4 ± 1.7	0.6 ± 0.8	3.8 ± 1.0	1.0 ± 0.0
Normoblast	11.4 ± 6.6	18.4 ± 4.2	17.3 ± 9.9	20.5 ± 11.4
CFU-GM	0.53 ± 0.21	0.03 ± 0.01	0.92 ± 0.43	0.04 ± 0.01
Cluster	0.58 ± 0.21	0.05 ± 0.03	0.86 ± 0.30	0.05 ± 0.02

[1] d = 1.064-1.061 g/ml

[2] d = 1.062-1.061 g/ml

Table III.

Composition of peak fraction obtained by isopycnic centrifugation in gradients with a density ranging from 1.085 to 1.050 g/ml (A; n = 7) and from 1.065 to 1.055 g/ml (B; n = 4).

cell suspension were underlayered as described under 'Methods'. Fractions of 2.5 ml were collected from the linear gradient. The fractions with a density of 1.0645 contained the highest number of cluster forming cells. The relative peak of CFU-GM was found in the fractions with a density of 1.0615 g/ml with an enrichment factor of 24.6 ± 7.4. The morphological composition of this peak fraction is presented in table III B. The recovery

	Fractions with density 1.065-1.057 g/ml
Nucleated cell	8.7 ± 3.2[1]
CFU-GM	88.8 ± 3.7
Cluster	80.3 ± 9.7
Myeloblast	58.6 ± 17.2
Promyelocyte	31 3 ± 10.5
Myelocyte	7.0 ± 3.0
Metamyelocyte	4 3 ± 1.5
Mature granulocyte	1.7 ± 1.2
Lymphocyte	6.3 ± 2.1
Monocyte	64.7 ± 19.7
Normoblast	12.3 ± 8.5

[1] The recovery of all fractions was put at 100%.

Table IV

Recovery (%) of different cell types in fractions obtained by isopycnic centrifugation in linear gradients with a density ranging from 1.065 to 1.055 g/ml (n = 3).

of the different cell types in the fractions of the shallow gradient which contained the clonogenic cells is shown in table IV. The fractions underlying the shallow gradient (d ≧ 1.065/ml) contained 84.3 ± 8.3% of the nucleated cells, 5.9 ± 5.2% of the CFU-GM and 13.3 ± 12.9% of the cluster forming cells.

Reduction of the number of lymphocytes by counterflow centrifugation followed by density gradient centrifugation

The interphase fraction from a discontinuous Ficoll-Isopaque gradient with a density of 1.085 g/ml was introduced into the counterflow centrifuge as described under 'Methods'. Lymphocytes, being the smallest nucleated human bone marrow cells, leave the separation chamber first, followed by small normoblasts. The appearance of the normoblasts is visualized by a distinct peak on the display screen of the scatter unit. In an attempt to eliminate the lymphocytes from the progenitor cell-rich fraction, bone marrow was separated in two different fractions monitored by the scatter device. The composition of the large-cell fraction is shown in table V. This fraction

	Large-cell fraction		Peak fraction (d = 1.0615 g/ml)	
	%	enrichment factor	%	enrichment factor
CFU-GM	0.045 ± 0.011	2.3 ± 0.5	0.33 ± 0.09	17.4 ± 3.6
Cluster	0.091 ± 0.030	2.8 ± 1.4	0.50 ± 0.16	17.5 ± 1.6
Myeloblast	5.5 ± 1.3	2.3 ± 0.6	28.8 ± 7.4	11.5 ± 3.9
Promyelocyte	3.3 ± 2.6	2.0 ± 1.0	20.8 ± 7.2	15.3 ± 4.7
Myelocyte	8.8 ± 5.1	5.5 ± 3.5	13.0 ± 4.7	10.6 ± 6.7
Metamyelocyte	13.8 ± 5.1	1.0 ± 0.3	9.3 ± 5.6	1.5 ± 0.9
Mature granulocyte	49.0 ± 2.2	0.9 ± 0.1	5.0 ± 3.8	0.2 ± 0.2
Lymphocyte	0.5 ± 1.0	0.06 ± 0.10	0.5 ± 1.0	0.06 ± 0.1
Monocyte	5.5 ± 2.9	2.3 ± 0.8	12.0 ± 3.9	2.4 ± 0.6
Normoblast	11.3 ± 7.7	0.9 ± 0.2	4.0 ± 4.2	0.4 ± 0.01
Plasma cell	2.3 ± 1.0	1.6 ± 0.5	6.8 ± 4.3	6.1 ± 4.8

Table V.

Relative number (%) and enrichment of different cell types in a large cell size fraction obtained by counterflow centrifugation, and the fraction with the highest concentration of CFU-GM obtained by flotation centrifugation of the large cell size cells (n = 4).

contained 74.8% of the nucleated cells, 5,3% of the lymphocytes, and 94.9% of the CFU-GM. Further purification of the lymphocyte depleted fraction was carried out by flotation centrifugation in a continuous linear gradient of Percoll with a density ranging from 1.050 to 1.085 g/ml. Fractions of 3 ml were collected. The fraction with a density of 1.0615 g/ml was maximally enriched (17.5 times) for the myeloid clonogenic cells. Lymphocytes were hardly observed (table V). The three fractions with the highest number of CFU-GM contained 4.1 ± 0.8% of the original number of nucleated cells, 59.8 ± 8.4% of the CFU-GM and 0.2 ± 0.1% of the lymphocytes (n = 3). In another set of experiments described elsewhere (9) the absolute number of E rosette-positive cells in large cell fractions obtained by counterflow centrifugation of low density cells (d $\leq$ 1.070 g/ml) had decreased proportionally to the absolute number of lymphocytes, suggesting a nonselective removal of T-lymphocytes.

DISCUSSION

The main objective of this study to obtain a human bone marrow cell population enriched for CFU-GM was achieved by isopycnic centrifugation in Percoll gradients. The Percoll solutions were strictly maintained at an isotonic osmolality of 285 mosm/kg to avoid fluctuations in the density sedimentation profiles of the CFU-GM as described by Messner et al.(12). Many investigators subject the bone marrow to a preseparation procedure, e.g. hypotonic lysis (13), phagocytosis of iron (14) or dextran sedimentation (6). These procedures are time-consuming, nonreproducible, and responsible for lower recoveries of the myeloid progenitor cells. The recovery of the nucleated cells and progenitor cells after density sedimentation was not satisfactory due to the entrapment of the low-density cells in the pellet caused by shear forces exerted by the red cells during sedimentation. Application of bone marrow cells in Percoll solution with a density just at the highest density of the gradient at the bottom end followed by isopycnic flotation centrifugation improved the cell recovery. Only 6% of the nucleated cells and 1% of the original number of progenitor cells was lost in the pellet. An absolute and relative peak of the myeloid progenitor cells was found in the fractions with a density of 1.0615 g/ml. The enrichment of the CFU-GM in this peak fraction was 10 times. The colony- and cluster-forming cells appeared to have similar physical properties, although a tendency of the cluster-forming cells towards a higher density was noted. The myeloid progenitor cells formed a distinct narrow band in the density gradient as shown in table II: 81.7% of the CFU-GM were found in the fraction which contained only 10% of the nucleated cells. A shallow density gradient in the range of the density of the myeloid progenitor cell did increase the relative number of progenitor cells to almost 2% (table III) and the enrichment factor to more than 20. Morphologically, myeloblasts formed

the main population of the progenitor cell-rich fractions, but lymphocytes still represented an important subpopulation. Graft versus host disease (GVHD) is one of the major obstacles in allogeneic bone marrow transplantation (15) in which donor lymphocytes play a pathogenic role (16). Separation of lymphocytes from hematopoietic stem cells by bovine serum albumin gradients has not been very succesful till now (Dicke et al., 17). This is in agreement with our results. Lymphocytes are known to be smaller than human CFU-GM (18) and murine CFU-GM (19). For this reason we attempted to separate the lymphocytes from the CFU-GM by counterflow centrifugation (9), and this CFU-GM-rich fraction devoid of lymphocytes was subsequently fractioned by isopycnic flotation centrifugation. The peak fraction was 17.5 times enriched for clonogenic cells, contained almost 1% of clonogenic cells (table IV), and was highly enriched for myeloblasts. Only 0.2% of the original number of lymphocytes contaminated the three fractions with the highest number of clonogenic cells. These findings confirm earlier data (18, 19) that the committed progenitor cell population is found in a cell fraction with a cell size larger than the lymphocytic fraction. Opsonization of T lymphocytes in a bone marrow graft by treatment of the bone marrow with an anti-pan T-cell antibody (OKT-3) reduced the number of acute GVHD in allogeneic bone marrow transplantation (20). Korbling et al. (21, 22) succeeded in prevention of GVHD by an albumin density gradient purification of canine allogeneic hematopoietic stem cells obtained by leukapheresis. The transfused CFU-GM-enriched fraction contained 0.2% of the original number of mononuclear cells. Further studies including those on the pluripotent progenitor cells (CFU-GEMM), T-lymphocyte progenitor cells and on the different lymphocytic subpopulationsare needed. But the results of these studies may indicate a new approach to prevent or mitigate GVHD by reduction of the total number of lymphocytes in human bone marrow using physical separation methods based on counterflow centrifugation and isopycnic flotation centrifugation.

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CHAPTER IV

CELL SIZE MONITORED COUNTERFLOW CENTRIFUGATION OF HUMAN BONE MARROW RESULTING IN CLONOGENIC CELL FRACTIONS DEVOID OF IMMUNOCOMPETENT LYMPHOCYTES

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SUMMARY

Human bone marrow cells were fractionated by physical methods in order to obtain cell fractions enriched for clonogenic cells and devoid of immunocompetent lymphocytes. The bulk of erythrocytes was removed by isopycnic gradient centrifugation on Ficoll - Isopaque^{R} (d = 1.085 g/ml) and the majority of mature granulocytes on Percoll^{R}(d = 1.070 g/ml). The nucleated cells were separated into various fractions by means of counterflow centrifugation. Continuous monitoring of the effluent of the elutriator by a light scatter device improved the reproducibility of the separation profiles. Progenitor cells did not form a single distinct peak and the maximal enrichment factor was 8.5. Lymphocytes were eliminated almost completely from the progenitor cell rich fraction (both CFU-GM and BFU-E). Physical elimination of lymphocytes from human bone marrow may offer an alternative approach to the prevention of GVHD in allogeneic bone marrow transplantation.

INTRODUCTION

Human bone marrow consists of a heterogeneous cell-population with a variety of hematopoietic cells at different stages of maturation. This heterogeneity is essential for normal bone marrow cell production which is governed to a large extent by a number of cell to cell interactions. Various cell separation methods have been applied in order to obtain more homogeneous cell fractions (1,2,3,4), as needed for experimental studies about factors which regulate growth and maturation of the different bone marrow cell populations.
This study describes separation profiles of the myeloid (CFU-GM) and erythroid (BFU-E) colony forming cells as obtained with an improved counterflow centrifugation system, in which the output of the elutriator was continuously monitored for cell number and light scatter. The elimination of the immunocompetent cells from the progenitor cell rich fractions was analysed by E-rosette formation, by monoclonal anti-T antibodies, and by in vitro assays of CFU-GM and BFU-E.

MATERIALS AND METHODS

Preparation of bone marrow cells.

Normal bone marrow is collected in buffered acid-citrate dextrose (pH 7.0). Bone marrow particles are disrupted by repeated aspirations through 16 gauge needles and the suspension is filtered through a nylon filter (pore-

size 70 µm). The cells are washed in Ca^{++}/Mg^{++}-free Hanks'balanced salt solution (HBSS) for removal of plasma, fat, and spicules and resuspended in HBSS containing 5% (w/v) fetal calf serum (FCS). A number of 150 - 200 x 10^6 nucleated cells in 35 ml suspension is layered on 15 ml Ficoll-Isopaque (specific density : 1.085 g/ml; pH 6.6; osmolality : 300 mosmol/kg and centrifuged (500 x g) at 18° C during 20 minutes for removal of the red cells. The nucleated cells at the interphase are collected, washed and resuspended in 3 - 5 ml FCS. This fraction contains 76 ± 4% of the original nucleated cells, 49 ± 12% of the mature myeloid cells and 105 ± 21% of the CFU-GM (n = 14).

Preparation of low density cells (d ≤1.070 g/ml)

Linear Percoll^R gradients (35 ml, density range 1.050 - 1.085 g/ml) are prepared as described elsewhere (5). After centrifugation (500 x g; 18° C; 30 min) the fraction with a density lower than 1.070 g/ml is collected by a peristaltic pump at a rate of 2 ml/min.
This fraction contains 33 ± 1% of the nucleated cells, 83 ± 8% of the CFU-GM and 5 ± 1% of the mature granulocytes (n=7).

Counterflow Centrifugation

Counterflow Centrifugation (CC) is performed with a Beckman J2- 21C refrigerated centrifuge equipped with a Beckman JE-6 elutriator rotor with a standard separation chamber (Beckman Instruments Inc. Palo Alto, CA, U.S.A.). The rotor speed is adjusted exactly by instalment of a finescaled speedselector (one scale unit : 2.6 ± 0.15 rpm). A high resolution counter (120 HMZ pH 6667 Philips, Eindhoven, The Netherlands) allows an accurate rotorspeed control. A constant counterflowrate is obtained by a peristaltic pump (Masterflex-Cole-Parmer Instr. Chicago, Ill. USA), connected to a pulse flattening air-chamber. The elutriation system is disinfected by overnight incubation with 70% ethanol. HBSS supplemented with 1% (w/v) heat inactivated FCS is used as elutriation medium and maintained at 18° C. The output of the elutriator is continuously sampled by means of a T-drain and analyzed for cell number and light scatter by the electro-optical unit of an Hemalog D (Technicon Instr. Corp., Tarrytown, USA). The light scatter signals are accumulated and displayed on a ND-600 multichannel analyser (Nuclear Data Inc, Schaumberg, Ill. USA) (6). The specimens are introduced at a flowrate of 0.3 ml/min by means of an infusor into the elutriator rotor spinning at 2200 rpm and at a counterflowrate of 9 ml/min. Only thrombocytes, erythrocytes and small lymphocytes are eluted at this rotorspeed and counterflow rate. The rotorspeed is maintained at 2200 rpm throughout the whole procedure, while the counterflow rate is increased stepwise 1-2 ml/min whenever the number of elutriated cells detected by the electro-optical

system falls below 100 cells/sec. The collected volumes vary from 50-250 ml per fraction. An average of 17 fractions is obtained during each elutriation separation.

Morhological studies

Cell counts are performed with a Coulter Counter (Model ZF Coulter Electronics, Hialeah, Florida, USA). Slides of the fractions, obtained by density sedimentation and elutriation, are made with a cytocentrifuge and stained with May Grünwald-Giemsa. Differential counting of 200 cells is carried out by a single investigator (G.B.).

Granulocyte/macrophage colony forming cells (CFU-GM)-assay.

All cultures are performed in 2 ml volumes in 35 ml plastic petri dishes (Costar). Dulbecco's modified Eagle's medium (Flow) is used with 20% heat inactivated FCS from a preselected batch in 0.3% Bacto-agar (Difco). Nucleated cells (2 x 10^5 or less) are seeded per plate with 4% (w/v) colony stimulating factor derived from a GCT-cell line (GCT-CSF) (7) or from human placenta conditioned medium (HPCM) (8,9). In some experiments the double layer agar technique is used with 1 x 10^6 mononuclear peripheral blood cells from one donor in the feeder layer (10). Duplicate cultures are incubated at 37° C in a fully humidified atmosphere of 5% CO_2 in air for 10 - 12 days. Cultures are scored at 20 - 40 x magnification. Aggregates consisting of 40 and more cells are scored as colonies, and aggregates of 3 - 40 cells as clusters.

Erythroid colony forming cells (BFU-E)-assay

The basic culture technique is adapted from the method of Iscove et al. (11). Cell suspensions at a concentration of 0.25 - 1 x 10^5/ml are plated in substantially modified Dulbecco's medium prepared from powder (IMDM, Gibco formula 78-5220) (12) to which is added 0.8% methylcellulose (Fluka, Schweiz), 10^{-4} M α-thioglycerol, 10% deionized bovine serum albumin (13), 20% heat inactivated foetal calf serum of a preselected batch (Rehatuin), 20% leukocyte conditioned medium (14) and 2 Units sheep erythropoietin/ml (Step III, Connaught Med. Res. Lab., Toronto, Ont. Can.). One-tenth milliliters of this suspension are placed in flat bottomed microwells (Limbro, Titertex) and incubated at 37° C in a fully humidified, 5% CO_2-air mixture. Orange to red colonies of at least eight cells are scored as CFU-E in four microwells on the 7th day of culture. Orange to red bursts of at least three subclusters or one single aggregate of more than 300 cells are scored as BFU-E in the other four wells on day fourteen of the culture.

E - rosette formation

The nucleated cells of the different fractions are incubated with AET-treated sheep red blood cells (15) in presence of human complement. All nucleated cells rosetted by 3 or more red cells are considered positive.

Determination of cells reacting with monoclonal antibodies

Nucleated cells of the various bone marrow fractions are assayed by means of the conventional indirect immunofluorescence technique after application of fluorescinated goat anti-mouse-Ig (Cappel Lab. Cochranville, Penn., USA).Mature T cells were detected by antibody OKT3; T-cell subsets were evaluated by antibody OKT4 and OKT8 for inducer/helper and suppressor/cytotoxic activities (16,17). The OKT monoclonal antibodies were obtained from Ortho Diagnostics. Additional analysis is performed with a murine anti-T antibody WT1, which recognises a human pre-T lineage specific antigen (18). The WT1 monoclonal antibody was kindly donated by Dr W. Tax (Department of Nephrology, University Hospital Nijmegen). The percentage of cells binding monoclonal antibodies is determined by flowcytometry at a wavelength of 488 nm (Cytofluorograf 30, Ortho Instruments, Westwood, Mass., USA).

RESULTS

Cell-separation of normal bone marrow cells with use of counterflow centrifugation

Contaminating red cells present in the bone marrow suspension were removed by gradient centrifugation on a cushion of 1.085 g/ml Ficoll- Isopaque. The interphase fraction was introduced into the elutriator centrifuge and separated with constant monitoring of cell size and cell number by light scatter. A typical example of the composition of the obtained cell fractions is shown in figure 1. Two minor peaks of nucleated cells were observed representing a lymphocyte and a normoblast peak respectively and one major peak representing the mature granulocytes (fig. 1E). The CFU-GM formed a broad peak (fig. 1 B) and they were found in the fractions obtained at counterflow rates ranging from 14 to 27 ml/min, corresponding to median sized cells. The separation profile of the cluster forming cells was almost identical to that of the CFU-GM (not shown). The majority of the erythroid progenitor cells was recovered in the same fractions as the myeloid progenitor cells, but the BFU-E formed a more distinct population in the fractions obtained at a counterflow rate of 14 to 17 ml/min (fig. 1C). The recovery of the various cell types in each fraction is shown in fig. 1 D and 1E. Most striking was the almost complete separation of the small

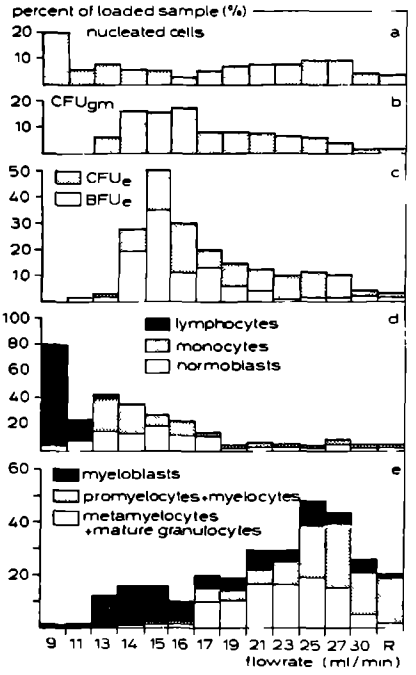

Fig. 1
Counterflow centrifugation of bone marrow cells with a density ≤ 1.085 g/ml. Example of one separation. The number per fraction was expressed as percentage of the number in the loaded sample (panel A/E).

lymphocytes from the committed progenitor cells. The first 2 fractions were almost pure lymphocytic (flowrates: 9 and 11 ml/min) followed by two distinct peaks of normoblasts and monocytes. Myeloblasts formed a subpopulation of the monocyte-rich fractions (flowrates: 13 to 16 ml/min). The different stages of maturation of the myeloid cells showed a smooth transition from one to the other fraction.
Table I represents the results of 9 subsequent counterflow centrifugation experiments. A peak fraction is defined as the fraction with the highest concentration of the concerning celltype in each separation. The counterflow rate of each peak fraction appeared to be remarkably reproducible (table I). The enrichment for CFU-GM and cluster forming cells was only modest and the enrichment was maximally 2.5 times. A relative increase of the percentages of progenitor cells was found in the fractions obtained at counterflow rates of 15 and 16 ml/min followed by a peak at 17 ml/min and a gradual decline at increasing counterflow rates. This phenomenon is well known from velocity sedimentation procedures and it probably reflects the

various cell cycle stages of the progenitor cells (19). The recovery of the nucleated cells was : 96 ± 4%; of the CFU-GM 106 ± 20% and of the cluster forming cells: 91 ± 19% (N=6). Reconstitution of all fractions after elutriation yielded a recovery of CFU-GM of 107 ± 16% (N=3).
The source of CSF for the cultures of all previous experiments was GCT-CSF. From studies of Boll et al. (20) it is known that different sources of CSF can stimulate subpopulations of murine CFU-GM differing in cell size and density. Parallel cultures were performed on the fractions obtained from 3 different CC separations. Three sources of CSF were used : GCT-CSF, HPCM-CSF and feeders with peripheral blood mononuclear cells. The culture profiles for the 3 sources of CSF were almost identical for both colony and clusterforming cells except for the tendency of GCT-CSF to stimulate the larger sized cells better (fig 2).

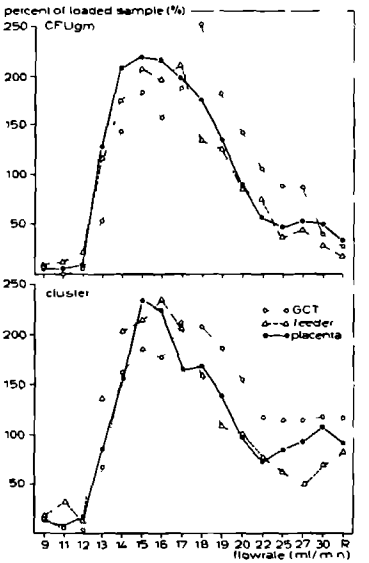

Fig. 2
Enrichment profiles of CFU-GM and cluster forming cells obtained by counterflow centrifugation of 1.085g/ml interphase cells, using 3 different sources of CSF: GCT (O·····O), feeders (▲------▲) and placenta (●——●).

Peak fraction*	Celltype	Bone marrow	Interphase	Peak fraction	
Flow rate		conc.	conc.	conc.	enrichment
ml/min		%	%	%	factor
9.3 ± 4.1**	Lymphocyte	8.6 ± 3.5	13.2 ± 4.5	93.9 ± 4.1	11.9 ± 3.4
14.1 ± 0.5	Normoblast	17.4 ± 7.2	14.7 ± 6.1	50.6 ± 16.7	3.3 ± 1.2
15.7 ± 1.1	Monocyte	3.5 ± 1.3	5.9 ± 2.0	30.8 ± 11.9	8.8 ± 3.6
16.1 ± 2.9	Myeloblast	1.4 ± 0.5	3.4 ± 1.6	13.6 ± 16.4	13.2 ± 7.8
16.0 ± 0.9	Cluster forming cell	0.029 ± 0.014	0.037 ± 0.010	0.089 ± 0.049	3.1 ± 0.7
17.0 ± 1.0	Colony forming cell	0.029 ± 0.011	0.040 ± 0.007	0.089 ± 0.023	3.1 ± 0.9
20.2 ± 1.1	Mature granulocyte	49.4 ± 7.3	41.6 ± 4.9	65.4 ± 5.1	1.4 ± 0.2
22.7 ± 0.8	Metamyelocyte	10.4 ± 2.4	10.1 ± 3.0	24.7 ± 6.5	2.5 ± 0.7
26.1 ± 2.6	Promyelocyte	0.9 ± 0.5	2.3 ± 1.5	13.7 ± 6.3	16.9 ± 6.3
26.1 ± 2.9	Myelocyte	6.4 ± 3.8	3.4 ± 1.6	13.6 ± 6.4	5.8 ± 3.1

*Peak fraction was defined as the fraction with the highest concentration of the concerning celltype.
**Mean ± standard deviation.

Table I.

Concentration and enrichment of different celltypes in the peak fractions, obtained by counterflow centrifugation (n = 9) of interphase cells (d ≦ 1.085 g/ml).

Peak fraction	Celltype	Bone marrow	Low density cells	Peak fraction	
Flow rate ml/min		conc. %	conc. %	conc. %	enrichment
10.6 ± 0.9*	Lymphocyte	7.3 ± 3.0	33.8 ± 14.8	92.5 ± 4.8	16.7 ± 6.5
14.8 ± 0.8	Normoblast	20.5 ± 1.7	24.5 ± 8.2	48.5 ± 8.7	2.4 ± 0.9
16.0 ± 1.6	Monocyte	3.5 ± 1.0	7.8 ± 2.2	28.3 ± 7.3	8.6 ± 3.3
19.8 ± 2.0	Colony forming cell	0.032 ± 0.011	0.105 ± 0.041	0.263 ± 0.076	8.5 ± 1.5
20.4 ± 1.5	Cluster forming cell	0.036 ± 0.013	0.086 ± 0.043	0.221 ± 0.078	5.9 ± 2.3
20.6 ± 1.5	Myeloblast	1.5 ± 1.0	6.3 ± 2.0	15.3 ± 5.2	11.4 ± 2.5
21.2 ± 1.3	Mature granulocyte	54.0 ± 2.7	8.8 ± 3.8	34.5 ± 6.8	0.7 ± 0.1
24.4 ± 1.9	Metamyelocyte	7.5 ± 4.2	4.0 ± 1.4	17.8 ± 6.2	3.5 ± 1.7
27.4 ± 1.9	Promyelocyte	1.0 ± 0.5	3.5 ± 1.3	17.0 ± 6.5	17.0 ± 6.5
27.8 ± 2.3	Myelocyte	3.5 ± 1.3	7.5 ± 1.7	40.8 ± 12.9	11.4 ± 2.5

*Mean ± standard deviation

Table II.

Concentration and enrichment of different celltypes in the peak fraction, obtained by counterflow centrifugation (n = 6) of low density cells (d ≤ 1.070 g/ml).

Elutriation of low density bone marrow cells (d ≦ 1.070 g/ml)

The small size of the separation chamber in the elutriator rotor limits the number of cells to be separated. To reduce the number of contaminating mature granulocytes bone marrow was separated in a continuous gradient, and the low density fraction (d ≦ 1.070 g/ml) was collected. The elutriation profile of the progenitor cells in this fraction (fig. 3) was similar to that of

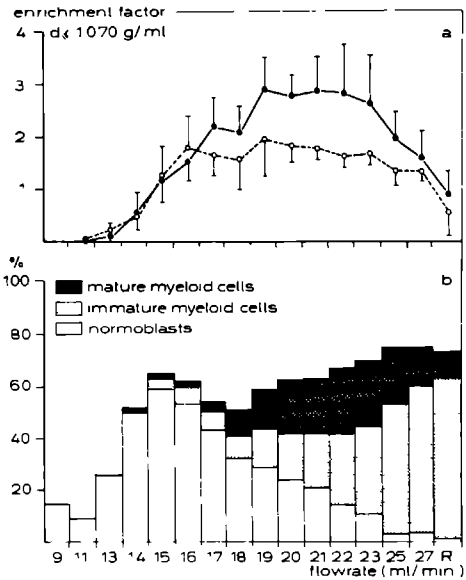

Fig. 3A
Enrichment of CFU-GM (●——●) and cluster forming cells (O---O) in fractions obtained by counterflow centrifugation of light density cells (d ≦ 1.070 g/ml).

Fig. 3B
Normoblasts: all nucleated red cells; immature myeloid cells: myeloblasts + promyelocytes + myelocytes; mature myeloid cells: segmented, band forms and metamyelocytes. The loaded sample was put at 100%.

the 1.085 g/ml interphase cells, but an additional increase of CFU-GM could be observed in the fractions obtained at flowrates ranging from 19 to 25 ml/min due to a selective removal of the mature granulocytes by density centrifugation. Table II presents the results of 6 separations. The sequence of the peak fractions of the different cell types has not changed apart from a shift of the myeloblast peak fraction from 16 to 21 ml/min. The enrichment factor for the CFU-GM obtained from the low density fraction has increased to 8.5 compared to 3 in the peak fraction obtained from bone marrow cells with a density $\leq$ 1.085 g/ml.

In an attempt to eliminate the lymphocytes from the progenitor cell rich fraction, light density bone marrrow cells were separated in 2 different fractions monitored by a scatter device. A very distinct lymphocyte peak was always observed. The appearance of a shoulder in the scatterhistogram indicated elutriation of the first normoblasts (fig. 4). As soon as such a

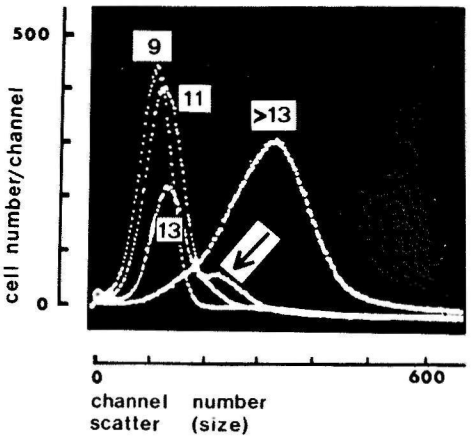

Fig. 4
Scatterhistograms of fractions obtained by counterflow centrifugation. Fractions 9 and 11 showed one single peak of lymphocytes. An additional peak (arrow) in fraction 13 visualized the appearance of normoblasts. Fraction > 13 contained the remaining cells (large cell fraction).

shoulder became visible on the display screen, the counterflow rate was not increased further until the number of cells in the effluent had fallen below 100 cells/sec. The obtained fractions were pooled and designated small size fraction containing: 95.1% of the lymphocytes, 3.0% of the CFU-GM, and 5.4% of the BFU-E (table III). The large cell fraction contained 53.2% of the nucleated cells from the loaded sample, 90.0% of the CFU-GM and 98.6% of the

	Small cell fraction	Large cell fraction
All nucleated cells	37.3 ± 6.9*	53.2 ± 5.5
Lymphocytes	95.1 ± 13.9	6.6 ± 3.1
Normoblasts	10.7 ± 3.2	105.0 ± 15.6
Myeloblasts	4.9 ± 4.3	85.1 ± 9.6
CFU-GM	3.0 ± 2.1	90.0 ± 15.6
Cluster forming cells	3.6 ± 2.6	88.0 ± 11.2
BFU-E	5.4 ± 8.0	98.6 ± 14.3
CFU-E	2.0 ± 2.2	94.5 ± 22.4
E rosette pos.cells	88.0 ± 22.0	4.8 ± 4.0

* Mean % ± standard deviation of loaded sample

Table III.

Recovery of nucleated cells and different celltypes in the small cell fraction (flowrate ranging from 7 to 10-12 ml/min) and the large cell fraction (flowrate > 10-12 ml/min) as monitored by scatter signal during elutriation of low density bone marrow cells ($d \leq 1.070$ g/ml)(n = 6).

Monoclonal antibodies	WT-1	OKT-3	OKT-4	OKT-8
Low density cells	7.0 ± 0.3*	20.4 ± 4.7	13.8 ± 4.8	8.3 ± 1.6
Small cell fraction	30.3 ± 3.2	53.6 ± 8.2	33.2 ± 6.8	22.8 ± 6.1
Large cell fraction	2.2 ± 1.2	3.2 ± 1.4	0.8 ± 0.7	2.4 ± 1.2

*Mean % ± standard deviation

Table IV.

Determination of the number of prae-T-lymphocytes (WT-1), mature T-lymphocytes (OKT-3), T-helper cells (OKT-4) and T-suppressor cells (OKT-8) in the low density fraction (d ≥ 1.070 g/ml) and the "lymphocytic" and "progenitor rich" fraction obtained by counterflow centrifugation (n = 4).

BFU-E. The contamination of this progenitor cell rich fraction with AET treated E-rosette positive cells was 4.8%. The viability of the progenitor cells was not compromised, as demonstrated by the complete recovery of BFU-E and CFU-GM (table III).

Determination of the presence of surface antigens for monoclonal T-antibodies was performed to confirm and extend the data on T-lymphocytes and T-cell subsets in these fractions. Table IV shows the distribution of the surface antigen positive cells in the low density fraction: WT1 (7.0 ± 0.3%), $OKT3^+$ (20.4 ± 4.7%), OKT4 (13.8 ± 4.8%) and $OKT8^+$ (8.3 ± 1.6%). The percentage positive cells in the lymphocyte rich fraction was: WT1 (30.3 ± 3.2%), OKT3 (53.6 ± 8.2%), OKT4 (33.2 ± 6.8%) and OKT8 (22.8 ± 6.1%). Monoclonal antibody binding in the progenitor cell rich fraction was only minimal (table IV.

DISCUSSION

Counterflow centrifugation provides a rapid and reproducible separation technique mainly based on differences in cell-size (21,22). Several adaptations were necessary to improve the stability of the rotorspeed and the flowrate (see Materials and Methods). An essential improvement was the connection of a scatter device to the effluent of the elutriator by means of a T-drain. Constant monitoring of cell scatter and cell number in the effluent

improved the reproducibility and enrichment factors of the different cellpopulations in peak fractions (see table I and II). Pretreatment of the marrow cells by a discontinuous gradient (Ficoll-Isopaque) with a density of 1.085 g/ml was found to be necessary for removal of erythrocytes. The complete recovery of the progenitor cells proved that the viability of the myeloid precursor cells was not compromised during the density sedimentation and counterflow centrifugation procedures. Counterflow centrifugation did not result in a single distinct peak highly enriched for CFU-GM. This might not be expected, as each cell population shows an increase in cell size as it passes through its reproductive cycle (23).

It appeared feasible to separate the lymphocytes almost completely from the committed progenitor cells. This is in agreement with the velocity sedimentation studies of Wells et al. (4). Further analysis of the obtained fractions for the presence of surface receptors for AET-treated sheep erythrocytes and monoclonal anti-T antibodies confirmed that the progenitor cell rich fraction was almost completely devoid of T-lymphocytes, both the suppressor and the helper subset. A recent study of Inoue et al. (24) on CFU-S in murine bone marrow fractions obtained by counterflow centrifugation showed that the average volume of cells in the peak fraction of murine CFU-S was 175 μm^3, whereas the average volume of murine lymphocytes was 100 μm^3. The human pluripotent hematopoietic progenitor cells (CFU-GEMM) (25) are expected to be present in fractions rich in small CFU-GM and BFU-E. If this is true, one may use counterflow centrifugation for the elimination of T-lymphocytes from donor bone marrow in an attempt to prevent or mitigate GVHD in allogeneic marrow transplantation.

In conclusion: these studies show that counterflow centrifugation provides a rapid and simple method for separation of human bone marrow cells with excellent recovery and viability. The separation is mainly based on cell size and therefore each celltype is separated depending on its cellcycle phase. Enrichment of CFU-GM and BFU-E was modest, but elimination of lymphocytes from the committed myeloid and erythroid progenitor cells was almost complete.

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CHAPTER V

CELL CYCLE SPECIFIC SEPARATION OF HUMAN BONE MARROW CELLS BY COUNTERFLOW CENTRIFUGATION AS MEASURED BY DNA-FLOW CYTOMETRY

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Submitted to Blood

SUMMARY

Human bone marrow was fractionated by counterflow centrifugation in 16 fractions with increasing cell size. Three distinct subpopulations could be recognized: small lymphocytic cells, medium-sized nucleated red cells and large myeloid elements. DNA-flowcytometry, ^{3}H-thymidine uptake and in vitro cell cultures showed that within the erythroid and myeloid cell fractions counterflow centrifugation separates the cellpopulations according to the cell cycle phase. Hypotonic treatment of bone marrow resulted in a complete abrogation of the proliferating erythroid cells. It appeared feasible to separate the small lymphocytic cells from the majority of BFU-E and CFU-GM, due to the larger size of the proliferating normoblasts and committed progenitor cells in comparison to the small lymphocytic cells.

INTRODUCTION

Human bone marrow consists of a variety of hematopoietic cell populations at different stages of maturation and cell cycle phase.
In vitro analysis of the hematopoietic cell growth regulation, the perturbance of hematopoietic cells by cytostatic agents and the separation of immunocompetent cells from the hematopoietic stem cells (HSC) requires separation techniques with minimal manipulation and optimal survival of the hematopoietic stemcells.
Counterflow centrifugation (CC) offers a rapid and gentle method with obvious advantages to other separation procedures like velocity sedimentation and rate zonal centrifugation (1,2,3,4). Continuous monitoring of the output of the elutriator-rotor for cell number and light scatter (5) made this separation method sensitive and reproducible.
This paper reports the data from cell fractions obtained by counterflow centrifugation of bone marrow, analysed for morphology, the presence of myeloid and erythroid progenitor cells and the cell cycle stages. Attention is drawn to the existence of 2 distinct proliferating populations representing the nucleated red cells and myeloid cells.
Physical separation of the lymphocytic cells from the HSC appeared almost complete, which may offer an approach to prevention of graft versus host disease (GVHD) in allogeneic bone marrow transplantation (BMT).
A rapid determination of the percentage S-phase cells by DNA- flow cytometry facilitates the discrimination of the lymphocyte rich fractions from the other cell fractions, as obtained by counterflow centrifugation.

MATERIALS AND METHODS

Preparation of bone marrow cells

Normal bone marrow from patients undergoing cardiac surgery is collected in buffered acid-citrate dextrose (pH 7.0). Bone marrow particles are disrupted by repeated aspirations through 16 gauge needles and the suspension is filtered through a nylon filter (pore-size 70 μm).
The cells are washed in Ca/Mg-free Hanks' balanced salt solution (HBSS) for removal of plasma and fat and then resuspended in HBSS containing 5 % (w/v) foetal calf serum (FCS).

Discontinuous density sedimentation

A number of 150 - 200 x 10^6 nucleated cells in 35 ml suspension is layered on 15 ml Ficoll-Isopaque (specific density: 1.085 g/ml; pH : 6.6; osmolality : 300 mosm/kg) and centrifuged at 500 g and 18° C for 20 minutes to remove the red cells. The nucleated cells at the interphase are collected, washed and resuspended in 3 - 5 ml FCS.
Light density cells ($g \leq 1.070$ g/ml) are obtained from linear gradients of Percoll with a density ranging from 1.050 - 1.085 g/ml as described before (6).

Counterflow Centrifugation

Counterflow Centrifugation (CC) is performed with a Beckman J2 - 21C refrigerated centrifuge equipped with a JE-6 elutriation rotor and a standard separation chamber (Beckman Instruments Inc. Palo Alto, Calif.). A constant counterflow rate is obtained by a peristaltic pump (Masterflex-Cole-Parmer Instr. Chicago, Ill. USA), connected to a pulse flattening air-chamber. The elutriation system is disinfected by overnight incubation with 70 % ethanol. HBSS supplemented with 1 % (w/v) heat-inactivated FCS, maintained at 18° C, is used as elutriation medium. The output of the elutriator is continuously sampled by means of a T-drain and analyzed for cell number and light scatter by the electro-optical unit of an Hemalog-D (Technicon Instr. Corp., Tarrytown, USA). A specimen containing 100 x 10^6 nucleated cells is introduced into the elutriator rotor spinning at 2200 rpm, at a counterflowrate of 9 ml/min, by means of an infusor (0.3 ml/min). Only thrombocytes, erythrocytes and small lymphocytes are eluted at this flow rate. The rotorspeed is maintained at 2200 rpm throughout the procedure and the counterflow rate is increased stepwise 1-2 ml/min whenever the number of elutriated cells, as detected by the electro-optical system, falls below 100 cells/sec. Cell counts of the different fractions are performed with a Coulter Counter (Model ZF Coulter Electronics, Hialeah, Florida, USA).

Morphological studies

Cytocentrifuge preparations of the fractions obtained by density sedimentation and elutriation are stained with May Grünwald-Giemsa. Differential counting of 200 cells is carried out by a single investigator (G.B.).

Granulocyte/macrophage colony forming cells (CFU-GM) assay.

All cultures are performed in 2 ml volumes in 35 mm plastic petri dishes (Costar). Dulbecco's modified eagle's medium (Flow Laboratories) is used with 20 % heat inactivated FCS from a preselected batch in 0.3 % Bacto-agar (Difco). Nucleated cells (2 x 10^5 or less) are seeded per plate with 4 % (w/v) colony stimulating factor derived from human placenta conditioned medium in the presence of endotoxin (7). Duplicate cultures are incubated at 37° C in a fully humidified atmosphere of 5 % CO_2 in air for 10 - 12 days. Aggregates consisting of 40 and more cells are scored as colonies and aggregates of 3-40 cells as clusters.

Erythroid colony forming cells (BFU-E) assay

The basic culture technique is adapted from the method of Iscove et al (8). The cell suspensions at a concentration of 0.25 - 1 x 10^5/ml are plated in a substantially modified Dulbecco's Medium prepared from powder (IMDM, Gibco, formula 78 - 5220) (9) to which is added 0.8 % Methylcellulose (Fluka, Schweiz), 10^{-4} M α-thioglycerol, 10 % deionized bovine serum albumin (10), 20 % heat inactivated FCS of a preselected batch (Rehatuin), 20 % leucocyte conditioned medium (11) and 2 units sheep erythropoetin/ml (Step III, Connaught Medical Research Laboratories). One-tenth milliliters of this suspension are placed in flat bottomed microwells (Limbro, Titertex) and incubated at 37° C in a fully humidified, 5 % CO_2-air mixture. Orange-to-red colonies of at least eight cells are scored as CFU-E in four microwells on the 7th day of culture. Orange-to-red bursts of at least three subclusters or single colonies of more than 300 cells are scored in four other wells on day fourteen of the culture.

DNA-flowcytometry

The nuclear DNA content of the cells is stained according to Krishan (12) by adding 0.5 x 10^6 cells to 10 ml hypotonic ethidium bromide (0.1 % trisodium citrate 25 mg/l ethidium bromide). The relative fluorescence of about 10^5 cells is measured with an ICP-11 Pulse Cytophotometer (Phywé, Göttingen, West Germany) at a flowrate of 400 cells/sec.
The percentages of cells in different phases of the cell cycle are calculated according to Baish (13).

Hypotonic shock

Interphase cells obtained from discontinuous density centrifugation are exposed to water for 15 seconds at 0° C (14). Isotonicity is restored with double strength Iscove DMEM.

Determination of ^{3}H-thymidine incorporation

Bone marrow samples are diluted in Medium 199 (Flow laboratories) buffered with 10 mM Tris (pH 7.4) and supplemented with 10 % heat inactivated fetal calf serum (Flow Laboratories) to a final cell concentration of 10^6 nucleated cells per ml. Four aliquots of 1 ml are incubated with 2 µCi ^{3}H-thymidine (specific activity 5 Ci/mmol) for 60 min at 37° C. The incubation is stopped by addition of 2 ml ice-cold medium with an excess of unlabeled thymidine. The cells are collected on glass fibre filters (Schleicher and Schüll no. 9), washed and lysed with water, and precipitated with 1 M perchloric acid (16). The total amount of radio-activity in the perchloric acid precipitated fraction is counted in a liquid scintillation counter (LKB 81000) with a counting efficiency of about 50 %.

RESULTS

Counterflow centrifugation of normal bone marrow cells with a density $\leq$ 1.085 g/ml

The interphase fractions with a density $\leq$1.085 g/ml were introduced into the elutriator rotor and the proliferation patterns of all collected fractions were measured with DNA-flow cytometry in 5 different experiments (fig. 1A). Two subsequent peaks of S-phase cells were observed in the fractions collected at counterflow rates of respectively 16 ml/min and 27 ml/min. Each S-phase was followed by an additional peak of G_2 + M cells obtained at a counterflow rate of 18 ml/min and 27 ml/min. The first peak of proliferating cells consisted mainly of nucleated erythroid cells whilst the second peak contained the immature myeloid cells (fig. 1B). In two additional experiments the proliferation patterns as measured with DNA-flowcytometry were compared with ^{3}H-thymidine-incorporation (fig. 2). No obvious discrepancies were observed.

Hypotonic lysis in distilled water for 15 seconds resulted in the removal of the nucleated erythroid cells from the 1.085 g/ml interphase fraction. A relatively high proportion of the immature erythroid cells possesses obvious proliferative activity as hypotonic treatment caused a decrease of the S-phase from 13.1 ± 1.6 % to 9.3 ± 1.1 % and of the G_2M-phase from 4.0 ± 1.1 to 2.3 ± 0.6 % (n=5). The results of counterflow centrifugation of these 5

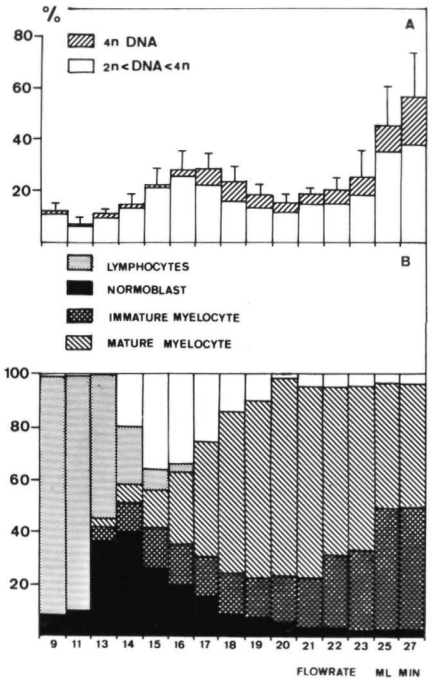

fig. 1
Counterflow centrifugation of bone marrow cells with a density ≦ 1.085 g/ml (n=5). Comparison of DNA-flowcytometry (panel A) with morphology. Panel A: 4n DNA: G_2 + M-phase-cells; 2n < DNA < 4n: S-phase cells (expressed as percentage per fraction). Panel B: each bar represents mean differential counts of a fraction with the four most important populations depicted. The remaining part of a bar is composed of mainly monocytes, plasmacells and megakaryocytes.

bone marrow samples, exposed to hypotonic lysis, are shown in figure 3. No proliferative activity was measured in the fractions with counterflowrates ranging from 11 to 18 ml/min due to removal of the nucleated red cells. The single peak of proliferating cells coincided with the fractions enriched for immature myeloid cells. In vitro assays of the myeloid erythroid progenitor cells were performed in all fractions obtained from CC. A typical example is shown in fig. 4. CFU-E and CFU-GM (scored at day 7 of culture) exhibited a peak in fraction 19 and 21, but the peak of BFU-E and CFU-GM (scored at day

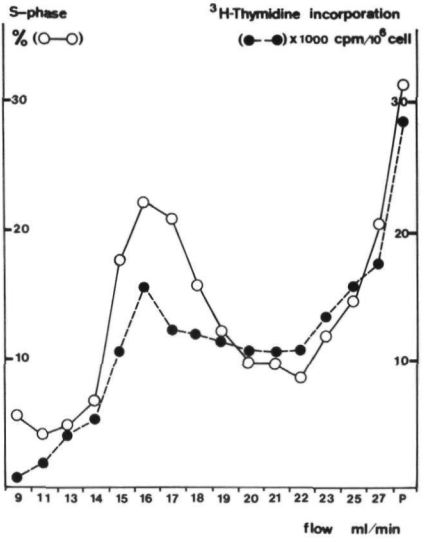

fig. 2
Comparison of the measurement of the percentage S-phase cells by DNA-flowcytometry and ^{3}H-thymidine uptake of bone marrow cells with a density 1085 g/ml separated by CC. (Example of one separation).

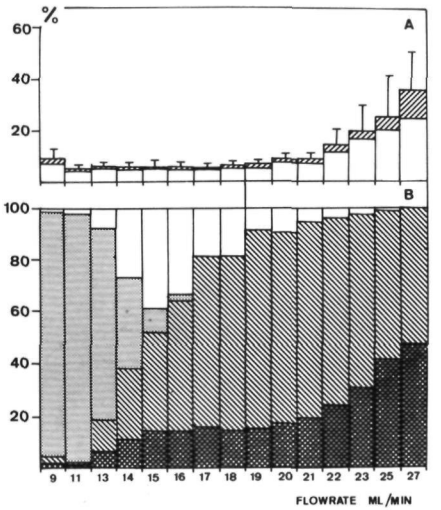

fig. 3
Counterflow centrifugation of bone marrow cells with density $\leq$ 1.085 g/ml treated with hypotonic lysis (n=5). For explanation of symbols see fig. 1.

14 of culture) appeared in the fraction obtained at a flowrate of 13 ml/min. This finding was very reproducible as shown by the results from 5 different experiments in table I. An almost complete separation of the lymphocytes and progenitor cells was observed (fig. 4c).

	peak fraction (ml/min)	
	day 7	day 14
CFU-GM	21.2 ± 1.0	14.8 ± 2.0
CFU-E/BFU-E	19.5 ± 3.4	13.5 ± 2.5

Table I.
Influence of in vitro culture time on the elutriation profile of myeloid and erythroid progenitor cells as measured by comparison of the fractions with the highest number of progenitor cells (peak fraction) obtained from 5 different experiments (mean ± SD).

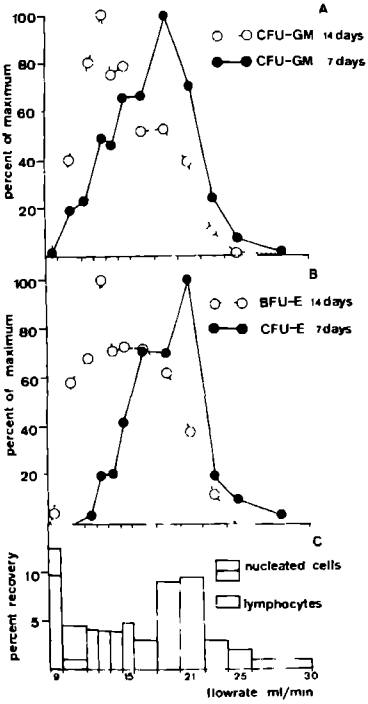

fig. 4
Profiles of myeloid (Panel A) and erythroid (Panel B) colony forming cells in day 7 (●——●) and day 14 (O-------O) cultures.
The number of colony forming cells in each fraction is normalised to the peak value (100%) and expressed as percent per ml/min.
The nucleated cell profile (panel C) shows the percent recovery of the loaded sample expressed as percent per ml/min.

Counterflow centrifugation of low density bone marrow cells (d $\leq$ 1.070 g/ml).

The small size of the separation chamber in the elutriator limits the number of cells to be separated. To reduce the number of mature nucleated cells the bone marrow was separated in a continuous density gradient. The low density fraction (d $\leq$ 1.070 g/ml) contained 25.4 $\pm$ 5.3 % of the nucleated cells, 3.2 $\pm$ 2.7 % of the mature granulocytes and more than 90 % of the myeloid and erythroid progenitor cells (n=5).
The percentage S-phase cells increased from 13.0 $\pm$ 3.4 % in the original sample to 20.6 $\pm$ 1.1 % in the low density fraction. Removal of the majority of the mature non-proliferating myeloid cells resulted in a disappearence of the dip between the two peaks of proliferating cellpopulations (fig. 5).

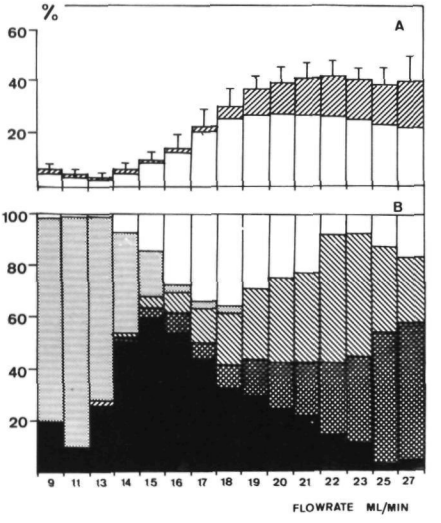

fig. 5
Counterflow centrifugation of low density bone marrow cells (d $\leq$ 1.070 g/ml). (n=5). For explanations of symbols see fig. 1.

It was also noticed that appearance of the progenitor cells in the effluent of the elutriator coincided with a rise in the percentage of S-phase cells due to appearance of the first proliferating normoblasts. In an attempt to separate the small lymphocytes from the hematopoetic stemcells 875 x 10^6 low density cells from a healthy bone marrow donor were introduced into the elutriator at a low counterflow rate of 5.5 ml/min (table II). From each fraction a sample was taken and analysed by FCM. A first increase of the percentage of S-phase cells was noted in the fraction obtained at counterflow rates of 10.5 ml/ min and a sharp increase in the fraction at a

Fraction ml/min	Lymphocytes %	Normoblasts %	S-phase-cells %
5.5	57	7	4.3
6.5	99	1	3.5
7	100	-	1.1
8	100	-	0.8
8.5	99	1	1.0
9	95	3	1.0
9.5	95	2	1.0
10	90	3	1.4
10.5	67	13	2.4
11	27	31	2.9
11.5	14	48	6.3
12	12	42	10.4
> 12	5	18	18.5
loaded sample	40	15	10.9

Table II.

Counterflow centrifugation of the low density cells (d < 1.070 g/ml) with small increments of flowrate. Relation of number of S-phase-cells with number of lymphocytes and normoblasts.

Fraction ml/min	Nucleated cells x 10^6	Lymphocytes x 10^6	AET-pos. x 10^6	CFU-GM x 10^4	BFU-E x 10^4
Loaded sample	875	350	280	55.3	53.6
5.5	63	36	26	0	0
6-10.5	394	366	201	0.7	0
11	40	11	16	1.9	2.5
> 11	380	19	7	44.8	36.5

Table III.

Number of nucleated cells, lymphocytes, AET-E-rosette positive cells and progenitor cells in 4 fractions obtained by counterflow centrifugation of low density cells (d ≤ 1.070 g/ml) with small increments of the flow-rate.

flowrate of 11.5 ml/min. At the same time a sharp decline of the number of the lymphocytes was observed. Despite the considerable number of cells loaded into the elutriator chamber,no progenitor cells were lost during introduction of the cells (see fraction: 5.5 ml/min, table III). The pooled fractions obtained at flowrates of 6-10.5 ml/min contained the majority of the lymphocytes and the fractions at flowrates >11 ml/min the majority of progenitor cells. At a flowrate of 11 ml/min a transition was found from the lymphocytes to the committed progenitor cells. In 6 experiments described elsewhere (16) low density ($\leq$ 1.070 g/ml) bone marrow cells were separated in 2 fractions with constant monitoring of light scatter and immediate analysation of the S-phase cells by FCM. The large cell fractions contained 4.8 ± 4.0 % of the original number of AET-treated E rosette positive cells, 90.0 ± 15.6 % of the CFU-GM and 98.6 ± 14.3 % of the BFU-E.

DISCUSSION

Counterflow centrifugation provides a rapid and reproducible separation technique mainly based on differences in cell-size (1,2). An essential improvement was the connection of a scatter device to the effluent of the elutriator by means of a T-drain. Pretreatment of the marrow cells by a discontinuous gradient (Ficoll-Isopaque) with a density of 1.085 g/ml was necessary for removal of erythrocytes. DNA-flowcytometry and ^{3}H-thymidine-uptake of the different fractions obtained by counterflow centrifugation revealed two peaks of proliferating cells: one mainly representing the proliferating immature erythroid cells and the other representing proliferating immature myeloid cells. Increase in cell size coincided with an increase of percentages S-phase and G2+M-phase cells of both the erythroid and myeloid series (fig. 1). Lysis of the erythroid nucleated cells resulted in a complete disappeareance of the first peak of proliferating cells and left one single peak of proliferating cells representing the larger immature myeloid cells. Counterflow centrifugation did not result in a single distinct peak highly enriched for CFU-GM. This might not be expected, as each celltype shows an increase in cell size when it passes through the replicative cycle (18). The velocity sedimentation studies of Dresch et al (19) are consistent with these findings: they found a population of larger clonogenic cells with a high percentage of S-phase cells and a population of smaller clonogenic cells with a low percentage of S-phase cells. This is in agreement with the shift of the progenitor peak to fractions with a lower flowrate, when the in vitro culture time was extended from 7 to 14 days. Analysis of the DNA-histograms of the different fractions, obtained by counterflow centrifugation, revealed a coincidence of the first appearance in the effluent of proliferating normoblasts and the committed progenitor cells. Careful elutriation of normal low density bone marrow cells (d $\leq$ 1.070 gr/l) with constant monitoring of the effluent for cell number and cell

scatter and additional DNA-flowcytometry made it feasible to discriminate the lymphocytes from the committed erythroid and myeloid progenitor cells. The committed progenitor cells are the immediate progeny of the HSC, but not identical to them. Murine CFU-S are more closely related or even identical to the HSC. A recent study of Inoue et al (20) on CFU-S in bone marrow fractions obtained by counterfow centrifugation showed that the average cell volume in the peak fraction of murine CFU-S was 175 μm^3, whereas the average volume of murine lymphocytes was 100 μm^3. If this difference in volume is also true for human bone marrow one may use counterflow centrifugation for the elimination of T-lymphocytes from donor bone marrow in an attempt to prevent GVHD in allogeneic marrow transplantation.
In conclusion: DNA-flowcytometry and ^3H-thymidine incorporation showed that counterflow centrifugation separates cells mainly based on cellsize and within each population on its cellcycle phase. Separation of the T-lymphocytes from the BFU-E and CFU-GM was almost complete.

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CHAPTER VI

DEPLETION OF IMMUNOCOMPETENT LYMPHOCYTES FROM HUMAN PLURIPOTENT PROGENITOR CELLS (CFU-GEMM) BY MEANS OF COUNTERFLOW CENTRIFUGATION

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SUMMARY

Counterflow centrifugation with continuous monitoring of the output for cell number and cell scatter was used to separate low density (d ≤ 1.070 g/ml) human bone marrow cells in two fractions: one containing the majority of small sized lymphocytes and the other the majority of the larger sized committed progenitor cells. The recovery of the pluripotent stem cells (CFU-GEMM) in the large cell fraction was complete (108%). The mitogenic reactivity of this putative stem cell fraction had decreased to 6 and 11%, as measured with phytohemagglutinin stimulation and one way mixed lymphocyte culture respectively.
Counterflow centrifugation appeared to offer a physical separation technique, by which the majority of the immune - reactive cells can be separated from the pluripotent hemopoietic stem cells.

INTRODUCTION

Various cell separation methods have been applied to obtain human bone marrow fractions devoid of immunocompetent cells in an attempt to prevent graft versus host disease (GVHD). Isopycnic sedimentation centrifugation in bovine serum albumin gradients (1), velocity sedimentation at 1 g (2), incubation with monoclonal anti-T antibodies (3) and differential agglutination with lectins have been described (4). Till now either these methods have failed to show a decrease in acute GVHD when applied to allogeneic bone marrow transplantation (BMT) in adults (5) or the low yield of hematopoietic stem cells (HSC) has limited its application to young infants (6).
Counterflow centrifugation offers another approach to the elimination of lymphocytic cells from human bone marrow. The separation takes place mainly depending on cell size (7). Continuous monitoring of cell size and cell number in the outflow of the separation chamber by a light scatter device made this separation method very reproducible and reliable (8). Lymphocytes could be eliminated almost completely from the committed progenitor cell rich fraction (8).
This paper describes an analysis of the different fractions obtained by counterflow centrifugation by means of in vitro culturing of pluripotent stem cells (CFU-GEMM) and assessment of T-lymphocyte function by in vitro stimulation with phytohemagglutinin (PHA) and by using mixed lymphocyte cultures (MLC).

MATERIALS AND METHODS

Preparation of bone marrow cellsuspensions

Normal bone marrow from patients undergoing cardiac surgery is collected in buffered acid-citrate dextrose (pH 7.0). Bone marrow particles are disrupted by repeated aspirations through 16 gauge needles and the suspension is filtered through a nylon filter (poresize 70 µm). The cells are washed in Ca^{++}/Mg^{++} - free Hanks' balanced salt solution (HBSS) for removal of plasma and fat and then resuspended in HBSS containing 5% (v/v) fetal calf serum (FCS). Assessment of the number of clonogenic cells and E rosette positive cells in whole bone marrow is performed after removal of red cells by using a discontinuous Percoll-gradient with a density of 1.085 g/ml. The recovery in 6 experiments was : nucleated cells : 91 ± 7%; mature granulocytes (segment and band forms) : 77 ± 8% and lymphocytes : 106 ± 19%.
The small size of the separation chamber in the elutriator rotor limits the number of cells to be separated. To reduce the number of contaminating mature granulocytes low density cell suspensions (d ≤ 1.070 g/ml) are prepared by isopycnic flotation centrifugation in Percoll gradients (8).

Counterflow centrifugation

Counterflow centrifugation (CC)is performed with a Beckman J2 - 21C refrigerated centrifuge equipped with a Beckman JE-6 elutriator rotor with a standard separation chamber (Beckman Instruments Inc. Palo Alto, Cal. USA). The output of the elutriator is continuously sampled by means of a T-drain and analyzed for cell number and light scatter by the electro - optical unit of the Hemalog D (Technicon Instruments Corp. Tarrytown USA) (9). Bone marrow specimens are introduced into the elutriator rotor spinning at 2200 rpm with a flowrate of 7 ml/min. Bone marrow is separated into a lymphocyte rich fraction and a progenitor cell rich fraction by increasing the counterflow rate stepwise 0.5 - 1 ml/min as described previously (8).

Granulocyte/macrophage colony forming cells (CFU-GM)-assay.

Nucleated cells (2×10^5 or appropriate dilutions) are cultured in 0.3% agar with 4% (w/v) colony stimulating factor (CSF), derived from human placenta, conditioned in the presence of endotoxin (10). Duplicate cultures are scored after 14 days of incubation. Aggregates consisting of 40 and more cells are counted as colonies.

Erythroid colony forming cells (BFU-E)-assay

The culture technique is adapted from the method of Iscove et al. (11). Cell suspensions at a concentration of 1 x 10^5/ml or less are plated in Iscove's modified Dulbecco's medium (IMDM, Gibco), to which is added 0.9% (w/v) methylcellulose (Fluka, Schweiz), 10^{-4} M thioglycerol, 10% (w/v) deionized bovine serum albumin (12), 20% (w/v) heat inactivated fetal calf serum of a preselected batch (Rehatuin), 20% (w/v) human leukocyte conditioned medium (HLCM) (13) and 2 units sheep erythropoietin/ml (Step III, Connaught Medical Research Laboratories). Duplicate 0.5 ml aliquots are cultured in 16 mm^2wells (Cluster^{24}, Costar). Orange to red bursts of at least 3 subclusters or one single colony of more than 300 cells are scored as BFU-E on day fourteen of culture.

Pluripotent colony forming cells (CFU-GEMM)-assay

Culture conditions, outlined by Fauser and Messner (14) with some modifications according to Ash et al. (15) are followed. The culture conditions are identical to those of the BFU-E assay, but HLCM has been replaced by 5% (w/v) human mononuclear leukocyte conditioned medium (PHA-HMCM) and the amount of erythropoietin has been reduced to one unit/ml. Mixed colonies are identified on day 16 of culture by their composite appearance. Individual colonies are removed by micropipetting into thin-tipped Pasteur pipettes, transferred onto slides and forcefully air-blown to spread the cells. Slides are stained with May Grünwald-Giemsa.

Mitogenic stimulation

PHA stimulation is determined as described elsewhere (16). Samples of 1 ml containing 3 x 10^5 nucleated cells are cultured in the presence of 25 µg PHA (Wellcome HA 15, Beckenham, England). Mixed lymphocyte cultures (MLC) are made by mixing 0.5 ml of a bone marrow fraction containing 1.5 x 10^5 nucleated cells with 0.5 ml of a lymphocyte suspension containing 1.5 x 10^5 irradiated (2000R) mononuclear cells of an unrelated healthy volunteer. Each fraction is tested against lymphocytes from 2 different volunteers. All cultures are carried out in triplo. The cultures to which PHA has been added are terminated after 3 days. The MLC's are harvested on day 6 of culture. DNA-synthesis is determined by addition of 0.25 µCi ^{3}H-thymidine (specific activity 5 Ci/mmol, Amersham, England) 24 hours before termination of culture. The cultured cells are collected on glass fibre filters (type AP 20.025.00, Millipore Corp. Bedford, Mass. USA). The dried filters are counted for 10 minutes (LKB 81000 Scintillation Counter) in 7 ml scintillation fluid. Fractionated bone marrow suspensions contain different numbers of spontaneously dividing cells. Results are therefore expressed as a mitogenic

response (MR) according to the formula:

$$MR = \text{DPM of stimulated cells minus DPM of spontaneously proliferating cells}$$

and as a stimulation index (SI) according to the formula:

$$SI = \frac{\text{DPM of stimulated cells}}{\text{DPM of spontaneously proliferating cells}}$$

(DPM = desintegrations per minute).
DNA-synthesis of spontaneously dividing cells is measured by incubation in medium without PHA, or with 1.5 x 10^5 autologous identical irradiated cells in the MLC.

E-Rosette formation

The nucleated cells of the different fractions are incubated with AET-treated sheep red blood cells (17) in presence of complement. All nucleated cells rosetted by 3 or more red cells are considered positive.

RESULTS

Low density bone marrow cells (d ≤ 1.070 g/ml) are separated in a small and a large cell fraction by means of counterflow centrifugation as described previously (8). The small cell fraction contains the majority of lymphocytes and a minor admixture of normoblasts and monocytes. The large cell fraction contains all myeloid cells and the remaining normoblasts and monocytes. In 4 experiments the presence of both the pluripotent and committed progenitor cells and the T lymphocytes was assessed. The recovery of the clonogenic cells in the low density fraction was complete (table I). The large cell fraction contained 86 ± 22% of the CFU-GM, 98 ± 17% of the BFU-E and 108 ± 30% of the CFU-GEMM (table I). Remaining E rosette positive cells in this fraction were 3 ± 2%. The lymphocyte rich (small cell) fractions contained about 3% of the clonogenic cells.
Mitogenic responses to PHA of the obtained marrow fractions were tested for several concentrations of PHA. The results of the optimal concentration (25 µg/ml)are presented in table II. Each bone marrow fraction contained

	Bone marrow (n=4)	Nucleated cells (d ≤ 1.085 g/ml)	Low density cells (d ≤ 1.070 g/ml)	Small cells	Large cells
		recovery (%)			
Nucleated cells x 10^6	296 ± 95*	86 ± 9	27 ± 5	7 ± 1	15 ± 2
Lymphocytes x 10^6	53 ± 8	95 ± 14	42 ± 8	30 ± 4	4 ± 3
E-Rosette pos.cells x 10^6	31 ± 14	100	44 ± 9	32 ± 5	3 ± 2
CFU-GM x 10^3	135 ± 32	100	120 ± 39	3 ± 4	86 ± 22
BFU-E x 10^3	221 ± 184	100	119 ± 18	2 ± 1	98 ± 17
CFU-GEMM x 10^3	21 ± 9	100	92 ± 13	3 ± 5	109 ± 30

* mean ± SD

Table I.

Recovery of clonogenic myeloid, erythroid and pluripotent progenitor cells in two fractions obtained by counterflow centrifugation of low density (d ≤ 1.070 g/ml) bone marrow cells. The number of nucleated cells and lymphocytes in the original bone marrow fractions was put at 100 %. The number of E-Rosette positive cells and clonogenic cells in the 1.085 g/ml interphase fraction was put at 100 %. Note : the low density fraction was obtained from whole bone marrow.

Bone marrow fractions	^{3}H-thymidine incorporation DPM x 10^3 / 3 x 10^5 nucleated cells			
	-PHA	+PHA	MR	SI
d ≦ 1.085 g/ml	7.4 ± 1.0*	17.3 ± 4.4	9.1 ± 3.2	2.1 ± 0.3
d ≦ 1.070 g/ml	13.4 ± 0.3	33.8 ± 4.2	17.2 ± 6.3	2.5 ± 0.3
"small" cells	1.0 ± 0.8	24.5 ± 11.3	23.2 ± 10.5	25.8 ± 12.1
"large" cells	13.3 ± 4.4	16.5 ± 5.5	3.3 ± 3.9	1.2 ± 0.4

*mean ± SD

Table II.

Mitogenic responses of bone marrow cells fractionated by isopycnic density sedimentation and counter-flow centrifugation by phytohemagglutinin (PHA : 25 µg/ml). For definitions of mitogenic response (MR) and stimulation index (SI) see "Materials and Methods" (n=4).

different numbers of spontaneously proliferating cells, as shown by incubation in medium without a mitogen for 3 days (table II). Results are expressed as mitogenic response (MR) and stimulation index (SI). Removal of the small lymphocytes from the low density fraction by counterflow centrifugation resulted in a considerable depletion of PHA responding cells in the remaining (large cell) fraction. The mitogenic response to PHA decreased from 17.2 ± 6.3 x 10^3 DPM /3 x 10^5 cells in the low density fraction to 3.3 ± 3.9 x 10^3 DPM in the large cell fraction. The stimulation index had fallen from 2.5 to 1.2. The lymphocyte rich fraction responded very well to PHA with a MR of 23.2 ± 10.3 x 10^3 DPM /3 x 10^5 cells and a SI of 25.8 ± 12.2 (table II). The calculated MR of all nucleated cells in the large cell fractions was 6 ± 11% of the MR in the unseparated bone marrow samples (table III).

Bone marrow fractions	Mitogenic response (x 10^3 DPM/3 x 10^5 NC)	Total mitogenic response (x 10^5 DPM)	(%)
d ≤ 1.085 g/ml	9.1 ± 3.2*	58 ± 33	100
d ≤ 1.070 g/ml	17.2 ± 6.3	51 ± 21	89 ± 15
"small" cells	23.2 ± 10.5	21 ± 11	35 ± 15
"large" cells	3.3 ± 3.9	4 ± 4	6 ± 11

* mean ± SD

Table III.

Mitogenic responses (MR) of bone marrow cell fractions obtained by isopycnic density sedimentation and counterflow centrifugation to PHA. Total MR of each fraction is calculated by multiplying the total number of nucleated cells (NC) and the MR / 3 x 10^5 NC. Last column shows the relative response of each fraction compared to that in the 1.085 g/ml interphase cells (n=4).

Bone marrow fractions	^{3}H-thymidine incorporation DPM x 10^3 / 1.5 x 10^5 nucleated cells			
	autologous	MCL	MR	SI
d ≤ 1.085 g/ml	2.3 ± 1.1*	4.4 ± 1.2	2.1 ± 0.3	2.1 ± 0.2
d ≤ 1.070 g/ml	4.6 ± 0.8	9.4 ± 3.2	3.5 ± 1.1	1.7 ± 0.2
"small" cells	2.5 ± 1.6	11.8 ± 1.4	9.2 ± 0.2	12.7 ± 8.3
"large" cells	10.6 ± 3.2	12.3 ± 4.0	1.7 ± 1.1	1.2 ± 0.1

*mean ± SD

Table IV.

Stimulation of bone marrow cells fractionated by isopycnic density sedimentation and counterflow centrifugation in mixed lymphocyte culture (n=3). For definitions of mitogenic response (MR) and stimulation index (SI) see "materials and Methods".

Spontaneous incorporation of ^{3}H-thymidine in fractionated bone marrow suspensions incubated with autologous identical irradiated bone marrow cells for 6 days varied considerably (table IV). The small cell fraction was highly stimulatory in the MLC with a MR of 9.2 ± 0.2 x 10^3 DPM per 1.5 x 10^5 nucleated cells and a SI of 12.7 ± 8.3. The large cell fraction showed a MR of 1.7 ± 1.1 x 10^3 DPM /1.5 x 10^5 nucleated cells and a SI of 1.2 ± 0.1. This fraction contained 12 ± 2% of the number of nucleated cells present in the starting bone marrow fraction. The calculated MR of all nucleated cells in this fraction was 11 ± 8% of the response of the total number unseparated cells (table V).

Bone marrow fraction	Mitogenic response (x 10^3 DPM/1.5 x 10^5 NC)	Total mitogenic response (x 10^5 DPM)	(%)
d ≤ 1.085 g/ml	2.1 ± 0.3*	35 ± 2	100
d ≤ 1.070 g/ml	3.5 ± 1.1	29 ± 4	78 ± 11
"small" cells	9.2 ± 0.2	16 ± 3	45 ± 9
"large" cells	1.7 ± 1.1	4 ± 3	11 ± 8

* mean ± SD

Table V.

Responses of bone marrow fractions obtained by isopycnic density sedimentation and counterflow centrifugation in mixed lymphocyte cultures. Total MR of each fraction is calculated by multiplying the total number of nucleated cells (NC) and the MR / 1.5 x 10^5 NC. Last column shows the relative response of each fraction compared to the response in the 1.085 g/ml interphase cells (n=3).

DISCUSSION

Counterflow centrifugation (CC) has been shown to offer a reliable and efficient technique for separation of human bone marrow based upon cell size (18). By continuous monitoring of the elutriator output with a light scatter device we were able to separate the majority of small lymphocytes (95%) from the large cell population (19). This progenitor cell rich fraction contained less than 5% of the original E^+, OKT_3^+ and WT_1^+ population (19). The WT_1^+ monoclonal antibody recognizes a human pre-T-lineage specific antigen (20). The recovery of committed progenitor population (BFU-E and CFU-GM) was about 80% (19). CFU-GM and BFU-E form the immediate progeny of

the hematopoietic stem cells (HSC) and are reliable indicators of bone marrow repopulation capacity of unseparated bone marrow (21), although the cell size of the HSC is not necessarily identical to that of the progenitor cells.

The human pluripotent hematopoietic stem cell (CFU-GEMM) gives rise in vitro to mature granulocytes, erythrocytes, macrophages, megakaryocytes and T-lymphocytes (22) and it has been shown to possess a limited self renewal capacity in vitro (15). This makes it a strong candidate for the pluripotent human HSC and comparable to the murine pluripotential stem cell : CFU-S. Separation of low density bone marrow cells into 2 fractions by CC resulted in a fraction of large cells with an apparent complete recovery of the CFU-GEMM and a contamination of 3 per cent of the original number E-rosette positive cells. Loss of the CFU-GEMM in the lymphocyte rich fraction was only 3%. The mitogenic stimulatory capacity of the stem cell rich fraction was investigated by means of PHA stimulation for 3 days and mixed lymphocyte culture for 6 days. The uptake of ^{3}H-thymidine measured in a PHA and MLC reaction of fractionated bone marrow samples can be profoundly influenced by extraneous factors in the culture system including accessory cells, such as monocytes, spontaneously proliferating myeloid cells and stimulatory clonogenic cells. This is shown by the varying spontaneous ^{3}H-thymidine uptake after 3 and 6 days of incubation (tables II and IV). The mitogenic response in the lymphocyte rich fraction was markedly increased, but it should be noted, that the MR to PHA in this fraction might be an underestimation, since the number of monocytes in this fraction was very low compared to the other fractions (mean 6%; range : 0-14%). The mitogenic response of the putative stem cell fraction was 6 and 11% measured with PHA stimulation and MLC-reactivity respectively (tables III and V). The major aim of this separation technique was to retain the full bone marrow repopulation capacity after removal of the majority of the lymphocytes. The definite proof will be provided by allogeneic bone marrow transplantation (BMT) with lymphocyte depleted bone marrow from HLA-identical, MLC-nonreactive siblings. The absolute number of lymphocytes in murine marrow grafts has been shown to be correlated with the incidence of GVHD (23). On the other hand several recent studies have shown a regeneration of OKT_3^+, E^+, Ia^- lymphocytes from an OKT_3^-, E^-, Ia^+ cell population (24). Also Messner et al. have shown the presence of T-lymphocytes in mixed colonies grown in the CFU - GEMM assay (22). Further clinical evaluation will be necessary to assess whether 95% removal of the small lymphocytes can mitigate or decrease the incidence of severe acute GVHD.

In conclusion: elimination of the majority of immune-reactive cells from the pluripotent hematopoietic stem cells appeared feasible by density equilibrium centrifugation followed by counterflow centrifugation. Constant monitoring of the output for light scatter improved cell size discrimination during elutriation separation considerably.

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CHAPTER VII

BONE MARROW REPOPULATION CAPACITY AFTER TRANSPLANTATION OF LYMPHOCYTE DEPLETED ALLOGENEIC BONE MARROW USING COUNTERFLOW CENTRIFUGATION

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SUMMARY

Bone marrow from six allogeneic HLA matched and MLC non-reactive siblings was fractionated by means of isopycnic flotation centrifugation (IFC) and subsequent counterflow centrifugation (CC). The low density fraction ($d \leq 1.070$ g/ml) obtained by IFC contained 20% of the nucleated cells and more than 90% of the myeloid and erythroid progenitors. The putative stem cell fraction obtained by CC showed a satisfactory recovery (88 %) of the CFU-GM and BFU-E and only 3.5% of the original number of T-lymphocytes.
Bone marrow repopulation capacity was not impaired in comparison to a comparable group of patients. Despite the average high age of this group (29.6 yrs), only one of the four evaluable patients developed GVHD.

INTRODUCTION

Graft versus host disease (GVHD) is one of the major drawbacks in allogeneic bone marrow transplantation (BMT) despite in vitro matching of donor and recipient by HLA-analysis and mixed lymphocyte cultures (1). Standard prophylactic therapy with methotrexate (MTX) does not prevent the occurrence of GVHD in the majority of allogeneic graft recipients (2), with an overall mortality of 30 percent or even higher if associated fatalities due to interstitial pneumonitis and infections are taken into account.
Several methods have been exploited to reduce the incidence and severity of GVHD, postulating that acute GVHD arises from immunologically competent mature T-lymphocytes present in the graft (3). Antilymphocyte globulin (4) and Cyclosporin A (5) are used to suppress the immune competent cells after marrow infusion. Another approach is selective elimination of the T-lymphocytes from the marrow graft before infusion. This was pioneered by Dicke et al.(6). Recently, in vitro incubation with purified anti-T sera (7) and anti-T monoclonal antibodies (8) have been reported.
This paper describes a physical method to separate the human lymphocytes from the committed myeloid and erythroid stem cells using density gradient centrifugation followed by counterflow centrifugation (9). Isopycnic flotation centrifugation is used to obtain a low density cell suspension devoid of the majority of erythrocytes and mature granulocytes. This low density cell suspension is subsequently subjected to counterflow centrifugation for separation according to cell size. We have shown that small sized lymphocytes can be separated from the majority of larger sized committed stem cells (9).
The results of isopycnic flotation centrifugation followed by counterflow centrifugation of donor bone marrow for allogeneic BMT are presented in this study. Analysis was performed by in vitro culture assays and the in vivo bone marrow repopulation capacity after allogeneic transplantation in six recipients.

MATERIALS AND METHODS

Patient characteristics

Patients in this study are subjects with acute non-lymphocytic leukemia (ANLL). Bone marrow remission had been induced by polychemotherapy (LAM-5 and LAM-6 protocol EORTC leukemias and hematosarcomas cooperative group). Six patients are studied and ages range from 18 to 36 years (mean: 29.5 ± 6.2 yrs). All bone marrow transplantations (BMT) are performed in first remission and the study represents consecutive BMT without any exclusions.

Transplant procedure

Bone marrow donors are HLA-A, B, C and D identical siblings and mutual nonreactivity in mixed lymphocyte cultures is demonstrated in all patients. Bone marrow remission (defined as less than 5% leukemic blasts in bone marrow) was confirmed in all patients. The transplant conditioning consists of cyclophosphamide 60 mg/kg/day for 2 days (days -6, -5) followed by 2 mercaptoethanesulfonate (Mesna^{R}) 28 mg/kg 0, 3, 6 and 9 hours after cyclophosphamide infusion. Fractionated total body irradiation (TBI) is administered using an 18 mEV Saturne linear accelerator on day -2 and -1. Irradiation was at 5.5 rad (5.5 cGy) per minute to a total midline dose of 900 Rad (9.0 Gy). Lung and eye shielding is used on the second day of irradiation from 770 Rad (7.7 Gy) onwards*. Bone marrow is infused intravenously 24 hours after completion of the TBI. Sterility of the marrow graft is confirmed by bacteriological studies**. Engraftment is documented using red blood cell antigens*** and karyotype analysis****. MTX is used as routine GVHD-prophylaxis for 102 days following the Seattle regimen (10). Patients are managed in single rooms with filtered air under positive pressure throughout the transplant period and all received oral selective gut decontamination including cotrimoxazole, colistin and miconazole. Hickman venous catheters are used for hyperalimentation throughout the acute post transplant period.

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Bone marrow aspiration

Bone marrow is collected under general or epidural anesthesia from the posterior iliac crests in disposable syringes containing 1 ml acid citrate dextrose formula- A with 50 U preservative free heparin. Syringe contents are injected directly into two 600 ml transfer packs (Fenwal) through a 70 µm

nylon filter fitted in a Swinnex-25 filter holder (Millipore). The transfer packs with the collected bone marrow are centrifuged (15 min; 500 g at 4° C) and supernatant fat and plasma are removed.

Isopycnic flotation centrifugation (IFC)

Isotonic stock solutions of Percoll ^{R} (Pharmacia, Uppsala, Sweden) are made by mixing 9 parts of Percoll with one part of 10 x concentrated HBSS. After determination of the remaining cell- and plasma-volume a 1280 ml bone marrow cell suspension is made with a density of 1.085 g/ml by adding appropriate amounts of Percoll stock solution and HBSS. A total number of 64 discontinuous Percoll gradients are prepared containing from top to bottom: 15 ml HBSS; 15 ml Percoll solution with a density of 1.070 g/ml and 20 ml cellsuspension with a density of 1.085 g/ml. The interphase fraction with a density $\leq$ 1.070 g/ml is collected after centrifugation at 500 g and 18 ^{0} C for 30 minutes. To reduce the volume the low density fraction is washed in HBSS and resuspended in 50 ml FCS and stored at 4 ^{0} C until use.

Counterflow Centrifugation (CC)

Counterflow Centrifugation is performed with a Beckman J2-21C refrigerated centrifuge equipped with a Beckman JE-6 Elutriation rotor with a standard separation chamber (Beckman Instruments Inc. Palo Alto, Calif.). The elutriation system is sterilised by gas evaporation apart from the separation chamber which is sterilised by autoclaving (20 min at 120° C). The system is assembled just before operational use inside a laminar downflow cabinet. The tubing and collection system outside the Beckman centrifuge are kept within the downflow cabinet throughout all separation procedures. A constant counter flowrate is obtained by a peristaltic pump (Masterflex-Cole-Parmer Instr. Chicago, Ill, USA). The pump is connected to a pulse flattening air-chamber. HBSS supplemented with 1% (v/v) heat inactivated FCS is used as elutriation medium and maintained at 18° C. The output of the elutriator is continuously sampled by means of a T-drain and analyzed for cell number and light scatter by the electro-optical unit of an Hemalog D (Technicon Instr. Corp. Tarry Town, USA). The light scatter signals are accumulated and displayed on a ND-600 multichannel analyser (Nuclear Data Inc., Schaumberg, Ill.USA). Specimens of 10 ml FCS containing 0.8 - 1.0 x 10^{9} nucleated low density cells are introduced into the elutriator rotor spinning at 2200 rpm and at a counter flowrate of 7ml/min by means of an infusor pump. Only erythrocytes and small lymphocytes are eluted at this flow rate. The rotorspeed is maintained at 2200 rpm throughout the procedure and the counterflow rate is increased stepwise 0.5 - 1 ml/min whenever the number of elutriated cells detected by the electro-optical system falls below 100 cells/sec.

Granulocyte-macrophage colony forming cells (CFU-GM) assay.

All cultures are performed in 2 ml volumes in 35 mm^2 plastic Petri dishes (Costar) using Dulbecco's modified Eagle's medium (Flow) with 20% heat inactivated FCS (Flow) from a preselected batch in 0.3% Bacto-agar (Difco). Nucleated cells (2 x 10^5 or less) are seeded per plate with 4% (v/v) colony stimulating factor (CSF), derived from human placenta, conditioned in presence of endotoxin (HPCM, 11). Duplicate cultures are scored after 10-12 days incubation. Aggregates consisting of 40 and more cells are counted as colonies.

Erythroid colony forming cells (BFU-E)-assay.

The basic culture technique is adapted from the method of Iscove et al (12). The cell suspensions at concentrations of 0.1 - 1 x 10^5/ml are plated in a substantially modified Dulbecco's Medium prepared from powder (IMDM, Gibco formula 78 - 5220) to which is added 0.8% Methylcellulose (Fluka, Schweiz), 10^{-4} M thioglycerol, 10% deionized bovine serum albumin (13) 20% heat inactivated foetal calf serum of a preselected batch (Rehatuin), 20% leukocyte conditioned medium (14) and 2 Units sheep erythropoietin/ml (Step III, Connaught Medical Research Laboratories). One-tenth milliliters of this suspension are placed in flat bottomed microwells (Limbro, Titertex). Orange-to-red colonies of at least eight cells are scored as CFU-E in four microwells on the 7th day of culture. Orange-to-red colonies of at least three subclusters or one single colony of more than 300 cells are scored as BFU-E in the other four wells on day fourteen of the culture.

E-rosette formation

The different fractions are incubated with AET-treated sheep red blood cells (15) in presence of human complement. All nucleated cells containing 3 or more red cells are considered positive.

DNA-Flowcytometry (DNA-FCM)

The nuclear DNA content of the cells is stained according to Krishan (16) by adding 0.5 x 10^6 cells to 10 ml hypotonic ethidium bromide (0.1 % trisodium citrate 25 mg/l ethidium bromide). The relative fluorescence of about 10^5 cells is measured with use of an ICP-11 Pulse Cytophotometer (Phywé, Göttingen, West Germany) at a flowrate of 400 cells/sec. The percentage of cells in different phases of the cell cycle are calculated according to Baish (17).

		Bone marrow	Low density fraction		High density fraction
			(%)		(%)
Nucleated cells	x 10^9	23.3 ± 5.6*	4.5 ± 0.7	20.0 ± 3.6	69.7 ± 9.3
Lymphocytes	x 10^9	3.5 ± 0.7	1.8 ± 0.3	50.4 ± 3.2	38.3 ± 7.8
E-rosette pos.cells	x 10^9	2.7 ± 1.2	1.2 ± 0.4	51.8 ± 20.7	ND**
CFU-GM	x 10^6	7.2 ± 3.8	7.4 ± 3.1	109.8 ± 32.6	3.6 ± 0.5
CFU-E	x 10^6	29.1 ± 11.1	25.4 ± 9.7	97.6 ± 44.5	ND
BFU-E	x 10^6	13.7 ± 9.0	9.2 ± 7.6	96.6 ± 36.7	ND

* mean ± SD

** ND: not determined

Table I.

Isopycnic flotation centrifugation in discontinuous Percoll gradients (n=5). Recovery in the low density fraction (d $\leq$ 1.070 g/ml) and loss in the high density fractions (d > 1.070 g/ml).

RESULTS

Preparation of low density cells

The results of isopycnic flotation centrifugation of bone marrow from 5 donors are presented in table I. Recovery of the nucleated cells in the low density fraction (d $\leq$ 1.070 g/ml) was 20.0 $\pm$ 3.6%. This fraction contained about 50% of the T - lymphocytes and almost all myeloid and erythroid progenitor cells. Loss of myeloid progenitor cells in the fraction with a density higher than 1.070 g/ml was 3.6 $\pm$ 0.5%. To exclude the possibility of inhibition by the high number of mature granulocytes (>75%) present in this fraction, samples of this fraction were subjected to a discontinuous sedimentation gradient of Percoll^{R} with a density of 1.070 g/ml. The recovery of nucleated cells was 3.2 $\pm$ 1.3%, and the yield of CFU-GM was 90.3 $\pm$ 26.7% (n=4) showing that inhibition by mature granulocytes was not likely. In one experiment bone marrow of a donor was separated in an intermittent flow cellseparator (Hemonetics 30) in order to obtain a low density fraction using discontinuous Percoll gradients in a closed system.

Counterflow centrifugation

Low density bone marrow cells were separated by counterflow centrifugation in 2 fractions in an attempt to remove the small sized lymphocytes from the hematopoietic stem cells (HSC). The small size of the separation chamber limits the number of cells to be separated. Therefore, specimens containing 0.8-1.0 x 10^9 low density cells were introduced into the elutriation system.

		Low density	"Lymphocytic"	"Stem cell"
Nucleated cells	x 10^9	4.9 $\pm$ 1.0*	1.7 $\pm$ 0.4	2.0 $\pm$ 0.3
Lymphocytes	x 10^9	1.7 $\pm$ 0.4	1.4 $\pm$ 0.5	0.12 $\pm$ 0.05
E-rosette pos.cells	x 10^9	1.2 $\pm$ 0.3	1.0 $\pm$ 0.3	0.04 $\pm$ 0.02
CFU-GM	x 10^6	7.9 $\pm$ 3.1	0.3 $\pm$ 0.2	6.5 $\pm$ 3.6
CFU-E	x 10^6	22.6 $\pm$ 12.2	0.2 $\pm$ 0.2	26.8 $\pm$ 14.6
BFU-E	x 10^6	12.6 $\pm$ 6.4	0.5 $\pm$ 0.4	10.1 $\pm$ 5 0

* mean $\pm$ SD

Table II.

Counterflow centrifugation of low density bone marrow cells from 6 donors. Separation into 2 fractions : a small cellsized "lymphocytic" fraction and a larger cellsized "stem cell" rich fraction.

This made three to four additional runs necessary. A distinct lymphocyte peak was always displayed by constant monitoring of the effluent for light scatter properties and by increasing the counterflowrate stepwise. This was followed by a separate peak indicating appearance of the first normoblasts in the effluent. At the same time small samples of the effluent were taken and analysed immediately for the presence of S-phase cells by means of DNA-flowcytometry (DNA-FCM). Samples containing lymphocytes only showed

		Bone marrow	"Stem cell" fraction recovery (%)
Nucleated cells	x 10^9	24.4 ± 25.7*	8.6 ± 1.4
Lymphocytes	x 10^9	3.7 ± 0.7	3.5 ± 1.0
E.rosette pos.cells	x 10^9	2.8 ± 1.0	3.4 ± 1.8
CFU-GM	x 10^6	8.1 ± 4.0	88.3 ± 35.3
CFU-E	x 10^6	25.4 ± 12.5	94.6 ± 62 8
BFU-E	x 10^6	15.3 ± 9 0	88.2 ± 64.7

* mean ± SD

Table III.

Recovery of lymphocytes and erythroid and myeloid progenitor cells in the "stem cell" rich fraction from bone marrow of 6 donors as obtained by isopycnic flotation centrifugation and counterflow centrifugation.

negligable numbers of S-phase cells. An increase of the percentage S-phase cells indicated the appearance of normoblasts in the effluent. Elutriation of the first normoblasts coincided with the appearance of myeloid and erythroid progenitor cells. Details of light scatter and flowcytometry studies are to be published in a separate paper. The rotor was stopped, as soon as a separate peak indicating normoblasts in the effluent became visible on the scatter display screen, confirmed by the appearance of S-phase cells in DNA-FCM. The contents of the separation chamber were collected and stored in autologous plasma until use. Data of the pooled lymphocyte and stem cell fractions are shown in table II. The loss of myeloid and erythroid progenitor cells in the lymphocyte rich fractions was less than 4 percent. The absolute number of lymphocytes and E-rosette positive cells contaminating the progenitor cell rich fraction was very low, respectively 3.5 ± 1.0% and 3.4 ± 1.8% (table III). The recovery of BFU-E and CFU-GM of the original donor bone marrow was higher than 85% (table III).

The ultimate proof of the presence of a sufficient number HSC in the marrow graft is delivered by the bone marrow repopulation capacity. Despite the low number of nucleated cells per kg present in the graft, bone marrow repopulation was not impaired, taken into account the influence of methotrexate GVHD-prophylaxis (table IV). Granulocyte-recovery of one patient and trombocyte recovery of 2 patients was not evaluable due to fatal infectious complications. One of the evaluable patients contracted GVHD grade II (18), which resolved with specific therapy. The clinical data will be presented in a separate paper.

	I	II	III	IV	V	VI
Nucleated cells x 10^7/kg	2.0	1.7	1.7	2.0	2.1	2.6
CFU-GM x 10^4/kg	9.7	6.1	2.9	12.6	15.9	8.0
BFU-E x 10^4/kg	11.0	27.9	6.6	13.2	9.8	14.9
Day:Granulocytes > 0.5 x 10^9/l	20	22	21	-[b]	19	16
Day:Thrombocytes > 20 x 10^9/l (without platelet support)	29	30	-[a]	-	26	19

a. patient died on day 22 after BMT from systemic aspergillus

b. patient died on day 8 after BMT from E-coli septicaemia

Table IV.

Number of nucleated cells and committed progenitor cells in marrow graft per kg body weight and its relation to bone marrow recovery.

DISCUSSION

Density profiles of the hematopoietic stem cells and the immunocompetent cells overlap considerably (9). This precludes density separation in allogeneic BMT of adult persons. It is generally accepted that the morphological appearance of the HSC is that of a small lymphocytic cell (19). Van Der Engh (20), using light scatter properties determined with a light activated cell sorter, estimated the diameter of the putative murine HSC (CFU-S) to be 7.2 µm whereas the size of thymocytes was calculated to be 6.0 µm. A recent study of Inoue (21), separating murine bone marrow by

counterflow centrifugation (CC) showed an average volume of CFU-S of 175 μm^3, and a mean volume of lymphocytes of 110 μm^3. This is in agreement with earlier studies of Van Bekkum (22). CC has been shown to provide a rapid and gentle separation technique mainly based on differences in cell size with excellent recoveries of human bone marrow cells without impairment of progenitor cell viability (23). Monitoring of cell number and cell size in the effluent continuously by means of a scatter device and the percentage of S-phase cells by FCM made it possible to separate lymphocytes almost completely from the committed progenitor cells (24).
The small size of the separation chamber of the elutriator and the large volume of donor bone marrow made a combination of 2 separation techniques necessary. Isopycnic flotation centrifugation in Percoll gradients yielded a low density fraction ($d \leq 1.070$ g/ml) of nucleated cells which contained more than 95% of the progenitors. This was due to the low viscosity and stability of the Percoll gradients and due to a better recovery after flotation centrifugation compared to sedimentation centrifugation (9). Preparation of a total number of 64 gradients and collection of the low density fraction from these gradients is laborious, time consuming and it carries a potential risk for infections. A method was developed to obtain low density cells with use of Percoll gradients in the Hemonetics 30 cell separator (patient 6). Details will be published separately. The recovery of committed progenitor cells after CC was satisfactory despite the high number of low density cells to be separated. The stem cell fraction contained 88% of the CFU-GM and BFU-E with 3.5% of the original number of T-lymphocytes contaminating this fraction.
Hematopoietic recovery could have been slowed down by a low number of HSC present in the graft despite a sufficient number of progenitors, but granulocyte and platelet recovery after BMT was comparable to that of a large group of patients similarly treated after BMT with methotrexate (25). Several clinical studies showed a successful prevention of GVHD by elimination of immunocompetent cells from the marrow graft (26, 27, 28). The low recovery of HSC by albumin gradient centrifugation (26) or lectin fractionation (27) hampered an application in adults. Only one of the four patients at risk showed signs of GVHD (grade II) despite the average high age of these patients (29.5 yrs).

In conclusion: a combination of density gradient centrifugation and counterflow centrifugation resulted in a cell fraction rich in hematopoietic precursors and depleted of the bulk of T-lymphocytes. Hematopoietic recovery after BMT was not impaired despite the low number of nucleated cells per kg in the graft.
This method may offer a new approach to prevention or mitigation of GVHD.

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SUMMARY

The prognosis of acute nonlymphoblastic leukaemia (ANLL) is mainly determined by the duration of a first complete remission. "Standard" polychemotherapy has failed to improve substantially disease free survival. Bone marrow transplantation (BMT) performed in first remission of ANLL, has lead to a disease free survival of 50-80 per cent at two years. Graft versus host disease (GVHD) is the major cause of early mortality after BMT, despite matching for the major histocompatibility complex and standard prophylaxis with methotrexate (MTX). Cytotoxic lymphocytes present in the marrow graft are assumed to be responsible for the GVH-syndrome. Removal of the immunocompetent T-lymphocytes from donor marrow has been exploited in several ways. This thesis describes a physical depletion of lymphocytes from human bone marrow using a combination of density centrifugation and counterflow elutriation (Chapter I).

Manipulation of human bone marrow prior to transplantation requires an accurate assessment of the number of hematopoietic stem cells (HSC). Committed myeloid and erythroid progenitor cells (CFU-GM and BFU-E) have proven to provide a reliable indication of the capacity to regenerate hematopoiesis after bone marrow ablative therapy. These progenitor cells proliferate in semisolid culture systems in the presence of specific growth regulators, such as colony stimulating factor (CSF) and erythropoietin (EPO). Standardization of the in vitro culture assays was improved by using a highly active CSF-source with a low level of inhibitors, by frozen marrow samples as an internal standard control and by estimation of peripheral nucleated cell contamination. Recently, an in vitro culture assay of the pluripotent stem cell has been developed, but its low number ($\leq$ 0.01%) in human marrow hampers a reliable quantification (Chapter II).

Density and size of the different celltypes in human bone marrow are the main physical characteristics, which can be used for separation of these heterogeneous populations. Colloidal silica coated with polyvinylpyrrolidone (Percoll^{R}) easily provides density gradients with constant physicochemical properties and low viscosity. Isopycnic separation with sample application to the bottom of continuous linear density gradients (flotation) improved the recovery and yielded a fraction 25 times enriched for CFU-GM. The average density of the fraction was 1.0615 g/ml. The fractions with a density ranging from 1.067 to 1.058 g/ml contained 10 per cent of the nucleated cells and 82 per cent of the CFU-GM (Chapter III).

Counterflow centrifugation (CC) provides a rapid and reproducible separation technique mainly based on differences in cell size. Constant monitoring of the effluent from the elutriator for cell number and light

scatter (∿ cell size) by a scatter device from the Hemalog D improved the reproducibility of the separation of different cell populations. CC did not result in a single distinct peak highly enriched for CFU-GM. However, it appeared feasible to separate the small lymphocytes almost completely from the median sized committed myeloid and erythroid progenitor cells, without a substantial loss of clonogenic cells. Further analysis of the obtained fractions for the presence of surface receptors for AET-treated sheep erythrocytes and monoclonal anti-T antibodies confirmed that the progenitor cell rich fraction was almost completely devoid of T-lymphocytes, both the suppressor and the helper subset (Chapter IV).

DNA-flowcytometry and ^{3}H-thymidine uptake of 16 different fractions obtained by CC revealed two separate peaks of proliferating cells: one mainly representing the proliferating immature erythroid cells and the other the proliferating immature myeloid cells. Increase in cell size coincided with an increase of percentages S-phase and G_2+M-phase cells of both the erythroid and myeloid series. The appearance of the first proliferating normoblasts in the effluent of the elutriator coincided with that of the committed progenitor cells. Careful elutriation with constant monitoring of the effluent for light scatter and additional DNA-flowcytometry improved the discrimination of the lymphocytes from the committed progenitor cells (Chapter V).

The human pluripotent hematopoietic stem cell (CFU-GEMM) gives rise in vitro to mature granulocytes, erythrocytes, macrophages, megakaryocytes and T-lymphocytes and it has shown a limited self renewal capacity in vitro. This makes the CFU - GEMM a strong candidate for the pluripotent human hematopoietic stem cell. The large cell fraction obtained by CC of low density bone marrow cells (d ≦ 1.070 g/ml) showed a complete recovery of CFU-GEMM (108%) with an admixture of 3 per cent of the original number E rosette positive cells. T-lymphocyte function was tested by in vitro stimulation with phytohemagglutinin (PHA) for 3 days and one way mixed lymphocyte cultures (MLC) for 6 days. The mitogenic response (MR) of the large stem cell rich fraction was 6 and 11 per cent of the MR of unseparated bone marrow as measured with PHA stimulation and MLC reactivity respectively (Chapter VI).

Bone marrow from six allogeneic HLA matched and MLC nonreactive siblings was fractionated by means of isopycnic flotation centrifugation (IFC) and subsequent counterflow centrifugation (CC). The small size of the separation chamber of the elutriator (4 ml) and the large volume of donor marrow made a combination of 2 separation technique necessary. IFC in Percoll gradients yielded a low density fraction (d ≦ 1.070 g/ml) with 20 per cent of the original number of nucleated cells and more than 95% of the progenitor

cells. The putative stem cell fraction obtained by CC showed a satisfactory recovery with 88% of erythroid and myeloid progenitor cells.
Since platelet and granulocyte recovery was comparable to that of a large group patients similarly treated with methotrexate after BMT, a significant loss of HSC seems to be excluded. Only one of the six patients showed signs of graft versus host disease (GVHD) despite the average high age of these patients (30 years) (Chapter VII).

GENERAL CONCLUSIONS

Combination of two physical separation techniques, based upon cell density and cell size, resulted in a cell fraction rich in hematopoietic clonogenic cells and depleted of 95 per cent of the original number of T-lymphocytes. Hematopoietic recovery after BMT was not impaired despite the low number of nucleated cells present in the marrow graft. Further clinical evaluation will be necessary to assess whether 95 per cent removal of small lymphocytes can decrease the incidence of severe acute GVHD.

Akute leukemie bij volwassenen is een ziekte die onbehandeld binnen 2 à 3 maanden tot de dood voert. Tegenwoordig kan m.b.v. celdelingsremmende geneesmiddelen (cytostatica) bij 75 procent van de patienten een komplete remissie bereikt worden. Dit betekent, dat bloed en beenmerg weer een normaal aspekt vertonen. Niet alle leukemische cellen blijken na de therapie te zijn opgeruimd en daarom treedt echter bij ca. 90 procent van de patienten toch een recidief op, meestal binnen één jaar na het bereiken van de remissie. Hierdoor is de 5-jaarsoverleving van deze ziekte slechts 10 procent, ondanks een intensieve nabehandeling. Eradikatie van alle leukemie-cellen, door bijvoorbeeld een totale lichaamsbestraling, zou leiden tot een onherstelbare beenmergbeschadiging. Door intraveneuze toediening van beenmergcellen van een weefsel-identieke broer of zuster na de bestraling kan dit voorkomen worden (allogene beenmergtransplantatie). Wordt deze transplantatie verricht in een eerste komplete remissie (voor het optreden van het recidief) dan wordt een twee-jaarsoverleving bereikt van 50-80 procent. Opmerkelijk is dat na 1 jaar na de transplantatie nauwelijks of geen recidieven van de leukemie meer optreden. Dit zou erop kunnen wijzen, dat bij een groot aantal sprake is van een definitieve genezing. De mortaliteit na de transplantatie wordt voornamelijk veroorzaakt door een afstotingsreaktie van immuun-reaktieve witte bloedlichaampjes (T-lymfocyten) aanwezig in het transplantaat en gericht tegen de gastheer: de zg. graft versus host disease (GVHD) of afstotingsziekte. GVHD kan grosso modo theoretisch op 2 manieren voorkomen worden òfwel door verwijdering van deze T-lymfocyten uit het donor-beenmerg voor de transplantatie òfwel door onderdrukking van de T-lymfocyten na de transplantatie. Dit proefschrift beschrijft een fysische scheidingsmethode waarbij m.b.v. centrifugatietechnieken gebaseerd op celdichtheid en celgrootte, T-lymfocyten uit het beenmerg verwijderd worden (hoofdstuk I).
De hematopoietische stamcel (HSC) vormt in het beenmerg via meerdere tussenstadia alle rijpe cirkulerende cellen in het bloed. Door de lage frekwentie (< 0.01%) is een exakte visuele schatting van het aantal HSC niet mogelijk. Echter de direkte afstammelingen van de HSC, de zg. voorlopercellen van de rode (erythroide) en witte (myeloide) reeks, kunnen in vitro gekweekt worden. De myeloide en erythroide voorlopercellen, gekweekt in halfvaste voedingsbodems, vormen een kloon van cellen en deze zijn als aggregaten herkenbaar onder de microscoop. Voor in vitro groei van de myeloide voorlopercel is een kolonie stimulerende faktor noodzakelijk; deze wordt door monocyten geproduceerd, maar ook bijvoorbeeld door placentaweefsel. De erythroide progenitorcel groeit in vitro o.i.v. erythropoietine (EPO) en een faktor geproduceerd door humane leukocyten geimmobiliseerd in agar (BPA : burst promoting activity). Onder invloed van EPO en een faktor geproduceerd door humane mononucleaire cellen (na

stimulatie door phytohemagglutinine) groeit een pluripotente, clonogene voorlopercel uit tot alle rijpe vormen die in het bloed en beenmerg aangetroffen worden. Mogelijk is deze cel identiek aan de HSC. Hoofdstuk II beschrijft deze cloneringsmethodieken en de wijze waarop de reproduceerbaarheid van deze bepalingen verhoogd werd.

De celdichtheid verschilt aanzienlijk voor de verschillende hemopoietische celtypen. Van deze eigenschap kan gebruik worden gemaakt om beenmerg te verrijken o.a. voor de hematopoietische stamcel. Kolloidale silicagel omgeven door een laagje polyvinylpyrrolidone (Percoll^{R}) is niet toxisch voor de hematopoietische cellen en door zijn lage viskositeit en konstante fysisch-chemische eigenschappen uitstekend geschikt om dichtheidsgradienten te maken. De cellen werden gebracht in een Percoll suspensie met een dichtheid iets hoger dan die van de kontinue lineaire dichtheidsgradienten en deze suspensie werd onder de gradient aangebracht. Na centrifugatie (flotatie) bleek de opbrengst van de clonogene myeloide cellen groter dan bij de gebruikelijke methode, waarbij de cellen in medium bovenop de gradient aangebracht worden (sedimentatie). De fraktie met een gemiddelde dichtheid van 1.0615 g/ml was 25 maal verrijkt voor wat betreft de myeloide voorlopercel. De frakties met een dichtheid van 1.067 tot 1.058 g/ml bevatten 10 procent van het oorspronkelijk aantal kernhoudende cellen en 82 procent van de progenitorcellen (hoofdstuk III).

Scheiding op celgrootte geschiedt m.b.v. technieken gebaseerd op de bezinkingssnelheid van deeltjes, meestal in apart gevormde scheidingskamers (velocity sedimentation). Het grote nadeel is de lange tijdsduur van de scheiding (4-8 uur). De elutriator rotor heeft dit nadeel ondervangen : cellen in een kleine scheidingskamer worden blootgesteld aan een centrifugale, naar buiten gerichte kracht en een centripetale tegengestelde vloeistofstroom. Bij verlaging van de centrifugale kracht of een verhoging van de tegenstroom verlaten de kleinste cellen de scheidingskamer het eerst. Bij menselijk beenmerg bleken lymfocyten de eerste kernhoudende cellen te zijn die de rotor verlieten, gevolgd door de normoblasten en de overige beenmergcellen. De myeloide en erythroide clonogene cellen werden gevonden in de frakties van de middelgrote cellen, zonder dat er sprake was van een sterke verrijking (maximaal 3 x). Een belangrijk hulpmiddel bij deze scheiding was de kontinue registratie van de lichtverstrooiing (light scatter) van de geëlutrieerde cellen. Dit gaf informatie over de celgrootte tijdens de scheidingsprocedure. Mede hierdoor bleek het mogelijk beenmerg m.b.v. elutriatie te scheiden in twee frakties : een fraktie van kleine cellen met meer dan 95 procent van de lymfocyten en een fraktie van grotere cellen met meer dan 90% van de progenitorcellen en minder dan 5 procent van de lymfocyten. T-lymfocyten hebben als eigenschap om schape-erythrocyten aan zich te binden (zg. E-rosette) en specifieke oppervlakte-antigenen,

waartegen antistoffen ontwikkeld zijn. Zowel de E-rosette-vorming, als de specifieke anti-T-antistoffen bevestigden dat minder dan 5 procent van de cellen in de progenitorcel rijke fraktie T-lymfocyten waren (hoofdstuk IV). De celgrootte varieert sterk tijdens de celcyclus. Rustende (niet in cyclus zijnde) cellen zijn relatief klein en delende cellen zijn relatief groot. Het al dan niet verkeren in celcyclus kan gemeten worden aan de hand van het DNA-gehalte per cel m.b.v. DNA-flowcytometrie of aan de inbouw van de hoeveelheid ^{3}H-thymidine. Humaan beenmerg bestaat kwantitatief uit twee hoofdpopulaties: een erythroide en een myeloide. Bij elutriatie bleek er inderdaad sprake te zijn van twee aparte, prolifererende populaties. De prolifererende erythroide populatie werd gevonden in de fraktïes met middelgrote cellen en de prolifererende myeloide populatie werd gevonden in de fraktïes met de allergrootste cellen. Het bleek dat de eerste prolifererende erythroide cellen gelijktijdig met de eerste progenitor cellen geëlutrieerd werden. Hierdoor kon een nog betere scheiding van lymfocyten en progenitorcellen worden verkregen (hoofdstuk V).

Bij verdere evaluatie bleek ook de pluripotente clonogene voorlopercel voornamelijk teruggevonden te worden in de fraktie met de grotere cellen. De funktie van de T-lymfocyten in de verschillende frakties werd in vitro gemeten m.b.v. een mitogeen : phytohemagglutinine en in de gemengde lymfocytenkweek. De veronderstelde stamcel-fraktie bleek nog slechts 6 resp. 11 procent van de oorspronkelijke mitogene aktiviteit te bevatten (hoofdstuk VI).

Tenslotte werd het beenmerg (gemiddeld 1.2 liter) van 6 identieke donoren gescheiden voor een allogene beenmergtransplantatie. Eerst werden de grote meerderheid van de rode bloedlichaampjes (> 99.99%) en de meerderheid van de kernhoudende cellen (± 80%) verwijderd m.b.v. dichtheidscentrifugatie in gradienten met een dichtheid van 1.070 g/ml. Vervolgens werden de cellen op grootte gescheiden m.b.v. tegenstroom-centrifugatie. De duur van de gehele procedure was ca. 14 uur. De fraktie met de grote cellen werd aan de patient intraveneus toegediend. Deze fraktie bevatte ongeveer 12 procent van het oorspronkelijk aantal kernhoudende cellen, 5 procent van de T-lymfocyten en 88% van de voorlopercellen. De beenmergrepopulatie bleek ongestoord en bij slechts één patient trad een afstotingsziekte op (hoofdstuk VII).

ALGEMENE KONKLUSIES

Door kombinatie van twee fysische scheidingsmethodieken, gebaseerd op celgrootte en celdichtheid, werd een beenmergfraktie verkregen die sterk verrijkt was voor de hematopoietische stamcel met minder dan 5 procent van het oorspronkelijk aantal T-lymfocyten.

Ondanks het lage aantal kernhoudende cellen was het vermogen om het beenmergkompartiment wederom te bevolken niet afgenomen.
Alhoewel uit dierexperimenteel onderzoek gebleken is, dat er een nauwe relatie bestaat tussen de frekwentie en de ernst van de afstotingsziekte enerzijds, en het aantal T-lymfocyten in het transplantaat anderzijds, zal uit verdere klinische evaluatie moeten blijken of dit bij de mens ook het geval is.

Het is een schier onmogelijke taak om iedereen te bedanken die bijdragen geleverd hebben aan het tot stand komen van dit proefschrift en het onderzoeksproject (U.O.P. 79-1), waarvan de opdracht luidde : "Isolatie en cryopreservatie van hematopoietische progenitorcellen".
De cryopreservatie van beenmerg en buffy coat zou nooit mogelijk zijn geweest zonder de energieke inzet van Peter Geerdink, die alle vereiste kennis en apparatuur in huis gehaald heeft. Dienstplichtig officieren Frank van den Ouweland,Reinier Raymakers en Cees Groot waren immer druk in de weer met het afnemen en invriezen van beenmerg en buffy coat, hierbij geassisteerd door Marianne Prenen en Gaby Ticoalu.
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Els, dank je voor de supportive care.

CURRICULUM VITAE

De auteur dezes werd op 26 april 1947 te Nijmegen geboren. Op het Canisius College te Nijmegen behaalde hij in 1966 het Gymnasium β-diploma. Het doktoraal examen in de Geneeskunde legde hij af in 1972 aan de Universiteit van Nijmegen. Na het artsexamen (1974) werd hij arts-assistent op de afdeling Inwendige Ziekten van het Akademisch Ziekenhuis te Nijmegen. De opleiding in Nijmegen werd 1 jaar onderbroken door een verblijf als registrar op de afdeling Bloedziekten van het Queen Elisabeth Hospital, Adelaide, South Australia, alwaar hij voor de hematologie "gewonnen" werd. In 1980 volgde inschrijving als internist in het specialistenregister. Dankzij een subsidie van de Universitaire Onderzoek Pool verrichtte hij van september 1979 tot november 1982 onderzoek op het laboratorium van de afdeling Bloedziekten van het Akademisch Ziekenhuis in Nijmegen. Sedertdien is hij een min of meer vaste medewerker van die afdeling.

ABBREVIATIONS

AET = 2-aminoethylisothiouronium bromide
ALL = acute lymphoblastic leukemia
ANLL = acute nonlymphoblastic leukemia
BFU-E = burst forming unit-erythrocyte
Bl = blood
BM = bone marrow
BMT = bone marrow transplantation
BPA = burst promoting activity
CC = counterflow centrifugation
CFU-E = colony forming unit-erythrocyte
CFU-GEMM = colony forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte
CFU-GM = colony forming unit-granulocyte/macrophage
CFU-S = colony forming unit-spleen
CSF = colony stimulating factor
CSA = colony stimulating activity
DMEM = Dulbecco's modified Eagle's medium
DNA = deoxyribonucleic acid
DPM = desintegrations per minute
EPO = erythropoietin
FCM = flowcytometry
FCS = fetal calf serum
GCT = giant cell tumor
G_2+M = premitotic resting phase and mitotic phase
GVHD = graft versus host disease
Hb = hemoglobin
HBSS = Hanks' balanced salt solution
HLA = human leukocyte antigen
HLCM = human leukocyte conditioned medium
HMCM = human mononuclear cells conditioned medium
HPCM = human placental conditioned medium
HSC = hematopoietic stem cell
LPS = lipopolysaccharide
MHC = major histocompatibility complex
MLC = mixed lymphocyte culture
MR = mitogenic response
MTX = methotrexate
NC = nucleated cells
OKT_3 = monoclonal antibody reactive with pan-T lymphocyte
OKT_4 = monoclonal antibody reactive with T-helper lymphocyte
OKT_8 = monoclonal antibody reactive with T-suppressor lymphocyte
PHA = phytohemagglutinin
SI = stimulation index
S-phase = replication phase of DNA
TBI = total body irradiation
WT_1 = monoclonal antibody reactive with pan-thymocyte and pan-T lymphocyte

Stellingen

I

Verwijdering van meer dan 95 procent van de lymfocyten uit donorbeenmerg heeft geen merkbare invloed op het repopulerend vermogen bij gebruik ervan voor beenmergtransplantantie.

Dit proefschrift.

II

Verwijdering van de lymfocyten uit het beenmerg met tegenstroomcentrifugering (elutriatie) kan alleen dán optimaal geschieden, indien de grootte van de cellen die de rotor verlaten continu wordt gecontroleerd met behulp van 'cell-scatter' apparatuur.

Dit proefschrift.

III

S-fase-meting m.b.v. flowcytometrie is een additioneel hulpmiddel bij de scheiding van lymfocyten en stamcellen m.b.v. tegenstroomcentrifugatie, aangezien de voorlopercellen, gelijktijdig met prolifererende normoblasten, uit het beenmerg geëlutrieerd worden.

Dit proefschrift.

IV

Dat het hemoglobinegehalte van een beenmerg-aspiraat een afspiegeling is van de hoeveelheid perifere bloedbijmenging, wordt bevestigd door de negatieve correlatie welke wordt gevonden tussen de concentratie progenitorcellen in een aspiraat en het hemoglobine-gehalte ervan.

Dit proefschrift.

Holdrinet R.S.G., Egmond J. van, Wessels J.M.C., Haanen C., A method for quantification of peripheral blood admixture in bone marrow aspirates. *Exp. Hematol.* 1980; 8: 103.

V

Het gebruik van ingevroren monsters normaal beenmerg als standaard is van essentieel belang bij de interpretatie van de resultaten van in vitro kweken met beenmerg of fracties daarvan.

Dit proefschrift.

VI

Subsidieaanvragen en publicaties over celscheidingen met behulp van tegenstroomcentrifugering moeten roeien tegen de mode-stroom van de monoclonale antilichamen in.

Eigen waarneming.

VII

De door vele onderzoekers uitgesproken mening dat de hematopoietische stamcel morfologisch sterk overeenkomt met de lymfocyt, berust op een onzorgvuldige interpretatie van de literatuur.

Bekkum D.W. van, Noord M.J. van, Maat B., et al., Attempts at identification of hemopoietic stem cells in mouse. *Blood* 1971; 38: 547.

VIII

Polyvinyl gecoate silica gel (Percoll^{R}) moge niet toxisch zijn voor de hematopoietische stamcel, maar is dit wel voor de monocyt.

Eigen waarneming.

IX

Sedert de invoering van de partiële darmdecontaminatie en het gebruik van centraal veneuze catheters is de stafylococcus epidermidis de meest geïsoleerde verwekker tijdens koortsperioden bij patienten met ernstige granulocytopenie.

Eigen waarneming.

X

De behandeling van secundaire acute myeloide leukemie bij patienten onder de veertig jaar moet gericht zijn op inductie van een complete remissie, zo mogelijk gevolgd door een allogene beenmergtransplantatie.

Preisler H.D., Early A.P., Raza A , et al., Therapy of secundary acute nonlymphocytic leukemia with cytarabine. *New Engl. J Med.* 1983, 308 21.

Witte T. de, Blacklock H.A., Prentice H.G , et al., Allogeneic bone marrow transplantation in a patient with acute myeloid leukemia secundary to Hodgkin's disease. *Cancer* 1983, in the press.

XI

De geestelijk gehandicapte heeft dezelfde rechten op welzijns- en gezondheidsvoorzieningen als een geestelijk volwaardige.

Verklaring van de rechten der geestelijk gehandicapten. Algemene vergadering der Verenigde Naties. 20 december 1971

XII

Gestoord gedrag eist een zekere mate van intelligentie.

P.P.M. van der Schoot, persoonlijke mededeling.

XIII

Bij rheumatoide arthritis is het serumgastrine gehalte verhoogd ten gevolge van een verminderde capaciteit tot maagzuur-secretie. Bijt rheumatoide arthritis in de gewrichten en likt het de maag?

Witte T de, Geerdink P.J., Lamers C.B., et al., Hypochlorhydria and hypergastrinemia in rheumatoid arthritis *Rheum. Dis.* 1979, 38 14.

XIV

Wanneer een curatieve behandeling niet mogelijk is, dan behoeft dit niet het einde van actief medisch handelen te betekenen.

XV

Het aanwezig zijn van de faciliteiten voor het opvangen van patienten die een beenmerg-lethale bestraling ondergaan hebben, mag en kan niet gebruikt worden in een discussie over kernbewapening.

Abrams H.L , Kacnel W.E. von, Medical problemens of survivors of nuclear war. *New Engl J. Med* 1981, 305 1226

Caldicott H.M., The final epidemic. Physicians could never reverse the ravages of nuclear war. *The Sciences* 1982 16.

Nijmegen, 30 juni 1983

T.J.M. DE WITTE